# In vitro evaluation of ara-c sensitivity, growth factor responsiveness, proliferation and clonal origin of leukemic clonogenic cells

Netty van der Lely

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**Stellingen**

Netty van der Lely, 16 december 1996

Ter vermindering van kinetische resistentie verdient het de voorkeur de cytostatische behandeling van AML patienten te combineren met individueel geselecteerde groeifactoren.
-dit proefschrift-

Aangezien leukemische stamcellen langzaam proliferende cellen zijn,verdient het de voorkeur AML patienten langduriger dan nu gebruikelijk is te behandelen met chemotherapie in combinatie met groeifactoren.
-dit proefschrift-

Door middel van IdUrd labeling kan het percentage clonogene cellen in S-fase nauwkeuriger worden bepaald dan met de gebruikelijke thymidine suicide techniek.
-dit proefschrift-

De mobiliteit van cellen in semi-solide kweekbodems kan het moeilijk maken een uitspraak te doen over de clonale origine van deze cellen. "Single-cell" assays zijn een oplossing voor dit probleem.

AML patienten in eerste complete remissie verkrijgen door behandeling met allogene of autologe beenmergtransplantatie een betere ziektevrije overleving dan met intensieve chemotherapie.
-Zittoun et al., N Engl J Med 1995;332:217-

Een goede arts verstaat de kunst om samen met een patient en/of diens familie de grens van een menswaardig leven te bepalen.

Een goede ontslagbrief herkent men aan één A4-tje.

Wat baat er kaars of bril, als de uil niet zien wil.
-Spreekwoord ten tijde van Jan Steen-

De agressiviteit van mede-automobilisten is omgekeerd evenredig aan de grootte van de auto die men zelf bestuurt.

De producenten van lange broeken spelen onvoldoende in op de toegenomen beenlengte van Nederlandse vrouwen.

Het uitrijden van de Elfstedentocht zal met het oplopen van het startnummer in toenemende mate afhangen van het kluunvermogen van de deelnemende schaats(t)er.

# In vitro evaluation of ara-c sensitivity, growth factor responsiveness, proliferation and clonal origin of leukemic clonogenic cells

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Een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

**Proefschrift**

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des namiddags om 1 30 uur precies

door

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*Aan mijn ouders*

## Abbreviations

| | |
|---|---|
| AML | - acute myeloid leukemia = acute nonlymphocytic leukemia |
| ARA-C | - cytosine arabinoside |
| ARA-CDP | - Ara-C diphosphate |
| ARA-CMP | - Ara-C monophosphate |
| ARA-CTP | - Ara-C triphosphate |
| ARA-U | - uracil arabinoside |
| BFU-E | - burst forming unit-erythroid |
| BM | - bone marrow |
| BrdU | - bromodeoxyuridine |
| CC | - counterflow centrifugation |
| CdR-kinase | - deoxycytidine kinase |
| CFU-GM | - colony forming unit-granulocyte/monocyte |
| CFU-L | - leukemic colony forming unit |
| CI | - confidence interval |
| CML | - chronic myeloid leukemia |
| C-MPL | - thrombopoietin |
| CR | - complete remission |
| CSF | - colony-stimulating factor |
| DAB | - 3,3-diaminobenzidin tetrahydrochloride |
| dCTP | - deoxycytidine 5'triphosphate |
| DMSO | - dimethylsulfoxide |
| DNA | - deoxyribonucleic acid |
| EPO | - erythropoietin |
| FAB | - French-American-British Cooperative Group |
| FCS | - fetal calf serum |
| FCM | - flow cytometry |
| FITC | - fluorescein isothiocyanate |
| FL | - FLK-2/FLT-3 ligand |
| G-CSF | granulocyte colony-stimulating factor |
| G-PBS | - glucose-phosphate-buffered saline |
| G6PD | - glucose-6-phosphate dehydrogenase |
| GM-CSF | - granulocyte-macrophage colony-stimulating factor |
| HBSS | - Hanks' balanced salt solution |
| HGF | - hematopoietic growth factor |
| HPCM | - human placenta-conditioned medium |
| HPLC | - high-performance liquid chromatography |
| HRP | - horseradish peroxidase |
| HSA | - human serum albumin |
| HSC | - hematopoietic stem cell |
| HU | - hydroxyurea |
| $^3$H-TdR | - tritiated thymidine |
| $ID_{50}$ | - 50% inhibition dose |
| IdUrd | - iododeoxyuridine |
| IL | - interleukin |
| IMDM | - Iscove's modified Dulbecco's medium |
| ISH | - in situ hybridization |
| IUra | - 5-iodouracil |
| LTBMC | - long-term bone marrow culture |
| M-CSF | - macrophage colony-stimulating factor |
| MDS | - myelodysplastic syndrome |
| MoAb | - monoclonal antibody |
| NC | - nucleated cells |
| PB | - peripheral blood |
| PBS | - phosphate-buffered saline |
| PHS | - pooled human serum |
| PI | - propidium iodide |
| RFLP | - restriction fragment length polymorphisms |
| SCF | - stem cell factor |
| SD | - standard deviation |
| SEM | - standard error of the mean |

## Contents

# Chapter 1

*Introduction and plan of investigation*

## Introduction

### Normal and Leukemic Hematopoiesis

The production of normal blood cells takes mainly place in the bone marrow. A relatively small number of pluripotent stem cells is responsible for the production of large quantaties of mature blood cells. Stem cells can differentiate to committed progenitor cells which can proliferate and differentiate to neutrophils, lymphocytes, monocytes, basophils, eosinophils, macrophages, erythrocytes and platelets. In addition, they also can produce new stem cells (self-renewal). Normal hematopoiesis is tightly controlled by hematopoietic growth factors (HGFs) (Fig. 1). Both stimulators and inhibitors of hematopoiesis have been identified.[1-8]

Acute myeloid leukemia (AML) is considered to be the result of a clonal expansion of one single transformed cell.[9,10] Chromosomal abnormalities can be detected in the majority of the AMLs.[11] The origin of the leukemic clone may be either a pluripotent stem cell or a cell restricted to the granulocyte-monocyte pathway [9,10,12,13] Hematopoiesis in AML is characterized by an accumulation of immature blasts that fail to differentiate to functional granulocytes or monocytes. Normal hematopoiesis is suppressed by displacement of the normal hematopoietic stem cells by leukemic cells, by production of inhibitory factors, by direct cellular inhibition, or by a combination of these processes.[10,13]

Myelodysplastic syndrome (MDS) is characterized by dyshematopoiesis. One to three cell lines may be involved. As a result, patients will suffer from anemia, granulocytopenia and/or thrombocytopenia.[14] In time, the disease may progress to overt acute leukemia. The abnormal preleukemic clone is assumed to arise at stem cell level.[15] Like in AML, chromosomal abnormalities are not uncommon in MDS (25-50%).[16] Observations suggest that (pre)leukemic clones may be organized in a fashion similar to normal hematopoiesis, with leukemic stem cells generating large numbers of somewhat more mature leukemic 'end' cells with a limited proliferation capacity.[13,17,18] The proliferation and survival of leukemic progenitor cells is regulated by the same HGFs that control normal hematopoiesis.[18]

Based on morphology and cytochemistry, AML and MDS can be distinguished into subtypes. Table 1 and 2 show the classifications according to the French-American-British (FAB) nomenclature.[19,20]

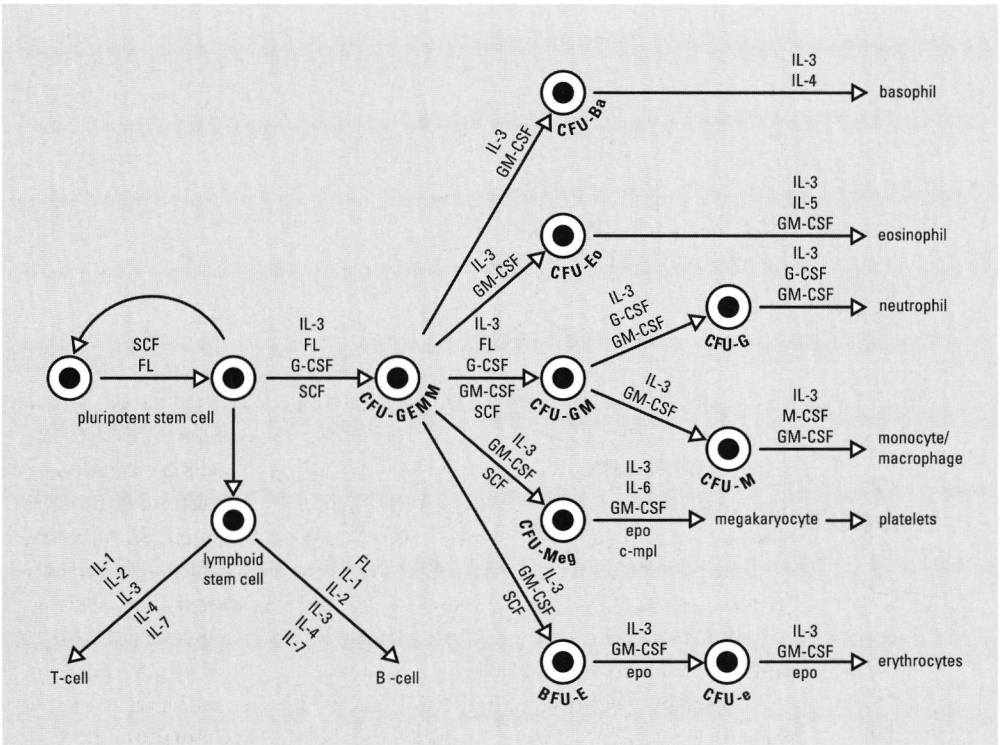



**Figure 1** Simplified scheme of normal hematopoiesis. For explanation of the abbreviations see list.

**Table 1** Morphological classification of AML according to FAB

| | |
|---|---|
| AML-M0 | acute myeloid leukemia without differentiation |
| AML-M1 | acute myeloid leukemia with minimal differentiation |
| AML-M2 | acute myeloid leukemia with differentiation |
| AML-M3 | acute promyelocytic leukemia |
| AML-M4 | acute myelomonocytic leukemia |
| AML-M5 | acute monocytic leukemia |
| AML-M6 | acute erythrocytic leukemia |
| AML-M7 | acute megakaryocytic leukemia |

**Table 2** Morphological classification of MDS according to FAB

| | |
|---|---|
| MDS-RA | refractory anemia |
| MDS-RARS | refractory anemia with ring sideroblasts |
| MDS-RAEB | refractory anemia with excess of blasts |
| MDS-CMML | chronic myelomonocytic leukemia |
| MDS-RAEB-*t* | refractory anemia with excess of blasts in transformation |

## In Vitro Growth of Progenitor Cells

**Growth factors** Normal and leukemic progenitors can be cultured *in vitro*. Before the identification and production of HGFs, conditioned media and feeder layers were used to stimulate cultures.[17] In the past years, studies showed that interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) not only support the growth of normal clonogenic cells, but also stimulate leukemic colony-forming cells in the majority of AML patients.[21-23] More recently, stem cell factor (SCF) has been identified as a regulatory molecule in both normal and leukemic hematopoiesis.[24-26] Other cytokines like macrophage colony-stimulating factor (M-CSF), interleukin-5 (IL-5), interleukin-6 (IL-6), and interleukin-11 (IL-11) exert less pronounced effects on myeloid leukemic cells.[22,27-29]

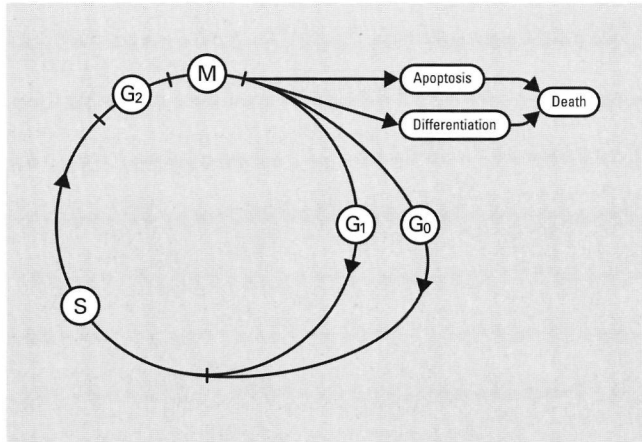
**Growth characteristics** *In vitro* cultured progenitor cells of normal bone marrow produce large colonies consisting of 50 to >10,000 differentiated cells. In contrast, AML progenitors exhibit aberrant growth patterns predominantly consisting of small clusters (maximum cluster size, 20 cells) or large clusters (maximum cluster size, 40 cells). Less frequently, leukemic colonies of >50 cells are cultured or AML clonogenic cells appear to be nongrowing *in vitro*.[30] Some leukemias can be cultured *in vitro* without the addition of exogenous growth factors. These spontaneously growing AMLs may produce their own growth factors (autocrine growth)[31] or may respond to low levels of growth factors produced by accessory cells such as monocytes of T-lymphocytes.

AML aggregates consist mainly of morphologically immature cells. Since this is not always the case, additional techniques like karyotyping, surface marker studies, and glucose-6-phosphate dehydrogenase (G6PD) analysis have been developed to establish the leukemic origin of cultured progenitor cells.[9,10,32,33]

## The Cell Cycle

Proliferating cells need to traverse through the cell cycle. Several phases can be distinguished (Fig. 2). The interval between the mitotic phase (M-phase, during which cell division takes place) and the S-phase is called $G_1$-phase. During S-phase, DNA synthesis results in a doubling of the cellular DNA content. The period between S-phase and mitosis is defined as the $G_2$-phase. Cells can be reversibly out of cycle (resting phase or $G_0$-phase). Terminally differentiated cells are irreversibly out of cycle. Both differentiation and programmed cell death (apoptosis) can lead to cell death. Apoptosis is characterized by DNA degradation into fragments and subsequent cell desintegration.[34]

**Figure 2** The concept of the cell cycle.




## Methods to Measure S-phase Cells

Several suicide techniques are available to determine *in vitro* the percentage of clonogenic cells in S-phase.[35-37] After short-term exposure to tritiated thymidine ($^3$H-TdR), hydroxyurea (HU), or cytosine arabinoside (Ara-C), the fractional reduction in cloning efficiency is considered to reflect the proportion of colony-forming cells in S-phase. The estimation of progenitors in S-phase by suicide techniques may not always be accurate.[36,37] Non-S-phase cells, probably late $G_1$ cells, can take up $^3$H-TdR and eventually will be killed.[36] This may result in an overestimation of the percentage of cells in S-phase. In addition, the confidence intervals of colony-forming assays are substantial. Statistically, this has a great impact on the accuracy of the suicide index, since calculation of the suicide index is based on the subtraction and division of colony numbers.

More recently, iododeoxyuridine (IdUrd) has become available for kinetic studies. This nonradioactive thymidine analog is incorporated into DNA during S-phase and can be detected by anti-IdUrd antibodies.[38,39] IdUrd can be used for both *in vitro* and *in vivo* studies.

## 1-ß-D arabinofuranosylcytosine (Ara-C)

Ara-C is a deoxycytidine analog with substantial cytotoxic activity against AML.[40] *In vivo*, Ara-C is rapidly deaminated by deoxycytidine deaminase to the inactive metabolite uracil arabinoside (Ara-U).[41] Ara-C is intracellularly phosphorylated to its monophosphate (Ara-CMP) by deoxycytidine kinase (CdR-kinase). The natural substrate for this enzyme, deoxycytidine, has a higher affinity for CdR-kinase than Ara-C. Phosphorylation of Ara-C to Ara-CMP is therefore influenced by the intracellular pools of deoxycytidine. Subsequent activation of Ara-CMP to Ara-CDP and Ara-CTP is facilitated by deoxycytidylate kinase and nucleoside diphosphokinase respectively. The

active metabolite Ara-CTP competes with deoxycytidine 5' triphosphate (dCTP) for binding to DNA polymerase and subsequent incorporation into cellular DNA.[42] The chain terminating activity of incorporated Ara-CTP results in inhibition of DNA synthesis. Hence, Ara-C is predominantly cytotoxic to S-phase cells. Consequently, cells not in S-phase may escape toxicity. This mechanism is called kinetic resistance

### In Situ Hybridization (ISH)

As stated earlier, the majority of the AMLs and up to 50% of the MDSs have chromosomal abnormalities.[11,16] Chromosomal aberrations can be detected by chromosome banding techniques(karyotyping).Unfortunately only metaphases can be analyzed with karyotyping. A low number of mitotic cells and selection of fast growing subpopulations may hamper this technique.[43] ISH overcomes these limitations. With nonradioactive chromosome-specific DNA probes, numerical and structural chromosome aberrations can be detected in both metaphase spreads and interphase nuclei.[44]

## Plan of Investigation

Although 60 to 70% of newly diagnosed AML patients achieve complete remission (CR) with chemotherapy, the majority eventually relapses and becomes more and more resistant to chemotherapy.[45] Non- or slowly cycling cells may escape chemotherapy through kinetic resistance. Metabolic resistance, for instance through P-glycoprotein expression is another mechanism of resistance to therapy.[46] Leukemic stem cells are held responsible for the maintenance of the leukemia. Hence, the aim of cytotoxic therapy is eradication of these leukemic stem cells with relative sparing of the normal bone marrow stem cells. Colony-forming assays can be used to study the effect of anti-cancer therapy on clonogenic cells. In AML, *in vitro* drug sensitivity appeared to be related to the *in vivo* complete remission rate.[47-49]

In *Chapter* 2, the cytotoxic effect of Ara-C on leukemic and normal clonogenic cells was investigated. Previous experiments demonstrated increased cytotoxicity with prolongation of the exposure time.[50] Therefore a prolonged (10-day) exposure period was chosen in an attempt to overcome the kinetic resistance of slowly cycling leukemic cells.

Clonogenic assays can also be applied to investigate the effects of growth factors on colony-forming cells. Growth factors may recruit cells into cycle and thus increase the toxicity of S-phase-specific agents. In *Chapter* 3 the effect of IL-3, GM-CSF, G-CSF, and combinations of these 3 factors was tested on blast cells of AML patients. Ara-C sensitivity was tested in the presence of weak and strong clonogenic cell growth-promoting hematopoietic factors.

The proliferative behaviour of progenitor cells can be investigated with suicide techniques. The described inaccuracy of these techniques made us search for new techniques. In Chapter 4, IdUrd was used to develop these techniques. The results were compared with the golden standard ($^3$H-TdR suicide technique).

The in Chapter 4 developed prolonged IdUrd labeling technique was applied in Chapter 5. With this technique, the influence of growth factors on the kinetics of leukemic and normal clonogenic cells could be investigated over a period of several days. Furthermore, the relation between the fraction of cycling CFU-L/CFU-GM and the Ara-C sensitivity was studied to investigate whether recruitment increased the toxicity of S-phase-specific agents.

The majority of AMLs and up to 50% of the MDSs have chromosomal abnormalities.[11,16] To establish the leukemic origin of progenitors more accurately, a novel interphase cytogenetic technique was developed to detect chromosomal aberrations in clonogenic cells (Chapter 6).

In Chapter 7, this in situ hybridization (ISH) technique was applied to study the clonal origin of AML at progenitor cell level during preleukemic phase, active disease, remission, and under in vitro culture conditions.

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# Chapter 2

## Prolonged exposure to cytosine arabinoside in the presence of hematopoietic growth factors preferentially kills leukemic versus normal clonogenic cells

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## Summary

We investigated the cytotoxic effect of the cell cycle-specific agent cytosine arabinoside (Ara-C) on clonogenic leukemic and normal bone marrow cells. To overcome kinetic resistance and to increase cytotoxicity, the cells were exposed to Ara-C in liquid culture medium for extended time periods, that is, 5 and 10 days. Subsequently the number of surviving clonogenic cells was determined in a semi-solid assay. All cultures were stimulated with the combination of interleukin 3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) to induce optimal cell proliferation. In comparison to normal clonogenic bone marrow cells (granulocyte-macrophage colony-forming units, CFU-GM) 5-day Ara-C exposure resulted in an equal to a slightly more effective kill of leukemic colony-forming cells (CFU-L) The Ara-C dose resulting in 50% inhibition ($ID_{50}$) was $1.6 \pm 1\ 6 \times 10^{-8}$ M for CFU-L ($n = 9$) and $6.7 \pm 4.3 \times 10^{-8}$ M for CFU-GM ($n = 4$, $p = 0.096$). Prolongation of the Ara-C exposure time from 5 to 10 days increased the cytotoxicity towards the majority of the leukemic clonogenic cells ($ID_{50}$: $0.8 \pm 0.6 \times 10^{-8}$ M) but not towards CFU-GM ($ID_{50}$: $5.7 \pm 2.8 \times 10^{-8}$ M). Overall, significantly more leukemic clonogenic cells than normal CFU-GM were killed after 10 days of exposure to Ara-C ($p = 0.039$). These results indicate that leukemic clonogenic cells can be eradicated preferentially by prolonged exposure to low dosages of Ara-C in the presence of hematopoietic growth factors with relative preservation of the normal hematopoietic progenitor cells.

## Introduction

The population of leukemic blast cells consists mainly of end cells and only a minority of stem cells, responsible for the maintenance of the leukemia.[1,2] In leukemic patients the aim of cytotoxic therapy is eradication of these leukemic stem cells with relative sparing of the normal bone marrow stem cells

Cytosine arabinoside (Ara-C), a potent antileukemic drug, is included in most acute myeloid leukemia (AML) treatment schemes. Its cytotoxic effect is thought to be determined mainly by the intracellular synthesis and retention of Ara-C triphoshate (Ara-CTP), which interferes with DNA synthesis.[3] For that reason Ara-C is preferentially cytotoxic to proliferating cells.[4,5]

*In vivo*, Ara-C is rapidly inactivated to uracil arabinoside (Ara-U),[6] so that Ara-C chemotherapy consisting of push injections or a few hour of infusion only results in short-term exposure of cells to Ara-C. Because a substantial number of leukemic stem cells is non- or very slowly cycling,[7-9] it is to be expected that these cells are not eradicated by the traditional Ara-C chemotherapy. Evidence for this kinetic resistence was found in our previous experiments.[10,11] In the presence of human placenta-conditioned medium (HPCM) as a source of colony-stimulating factor (CSF), clonogenic cells were exposed to Ara-C for 1 or 20 h. Even pharmacologically high concentrations of Ara-C only marginally affected the colony growth of normal bone marrow cells and leukemic blasts, reflecting the low number of cycling cells.[10] Only when the exposure time was prolonged to 5-day liquid incubation or 10-day continuous exposure in semi-solid medium (cocultivation), complete inhibition of granulocyte-macrophage colony-forming units (CFU-GM) and leukemic colony-forming units (CFU-L) was observed.[10,11] This indicated that during prolonged exposure clonogenic cells traversed the cell cycle.

With cocultivation, colony growth inhibition and not necessarily clonogenic cell kill is tested.[12] Furthermore, it is difficult to extend the results of the cocultivation test system to the clinical situation, where the outcome of chemotherapy does not depend on how many leukemic clonogenic cells can be detected at the end of the course but how many clonogenic cells still can grow out following cytotoxic drug exposure In this paper, therefore, we preferred to expose clonogenic leukemic and normal bone marrow cells to Ara-C in liquid culture for 5 and 10 days and subsequently assessed the number of surviving clonogenic cells in a semi-solid system. This test system would allow us to investigate whether prolongation of the Ara-C exposure time would lead to an increased toxicity towards CFU-L and how toxic this would be to normal CFU-GM. Cells were cultured in the presence of recombinant hematopoietic growth factores, which regulate the growth of both normal bone marrow cells and leukemic blasts.[13-16] Optimal growth was obtained with the combination of interleukin 3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF).

We observed that leukemic clonogenic cells were killed significantly more than their normal counterparts when they were exposed to Ara-C for 10 days in the presence of these three growth factors.

## Materials and Methods

**Patients** Bone marrow samples from nine previously untreated AML patients were used. According to the French-American-British (FAB) classification,[17]

five patients were classified as M2, three patients as M4, and one patient as M5. The samples contained 72%-94% blasts Selected leukemic samples requiring exogenous colony-stimulating factors to form aggragates in agar were used. Normal bone marrow was obtained from four patients undergoing cardiac surgery. Informed consent was given in all cases.

**Bone marrow collection, cryopreservation, and thawing** Bone marrow was collected in sterile buffered acid-citrate dextrose (pH = 7.0). Most of the red blood cells and mature myeloid cells were removed by centrifugation on a Ficoll (1.085 g/ml) density gradient (Sigma, St Louis, Missouri). One-milliliter vials containing 5-20 x $10^6$ interphase cells in Iscove's medium (Flow Laboratories, Irvine, Scotland), supplemented with 5% fetal calf serum (FCS; HyClone, Logan, Utah) and 10% dimethylsulfoxide (DMSO), were frozen in liquid nitrogen using a temperature-controlled freezer (Kryo 10; Planer Biomed, Sunbury, Middlesex, UK) Just prior to the experiment, cells were thawed rapidly in a 37°C waterbath, resulting in a cell recovery of >90%. The detailed procedure of freezing and thawing has been described before.[18]

**Incubation with Ara-C** Maximally $10^5$ leukemic and normal bone marrow cells per milliliter were suspended in Iscove's medium supplemented with 20% FCS, 50 IU/ml penicillin, and 50 µg/ml streptomycin (Flow Laboratories) and incubated in 15-ml culturing tubes (Falcon, Becton Dickinson, Lincoln Park, New Jersey). For our test system, optimal concentrations of recombinant human IL-3 (5 U/ml, kindly donated by Dr Wagemaker, ITRI/TNO, Rijswijk, The Netherlands), recombinant human GM-CSF (250 U/ml), and recombinant human G-CSF (250 U/ml, both kindly donated by Behring, Marburg, Germany) were added to the suspensions. The final Ara-C (Upjohn, Kalamazoo, Michigan) concentrations added varied from 5 x $10^{-9}$ to 5 x $10^{-6}$ M. Dilutions were prepared from freshly thawed Ara-C stock solutions. The drug was checked for concentration, purity, and stability by high-performance liquid chromatography.[6] In each experiment the 100% control growth was determined in duplicate. Cell suspensions were placed in an incubator (37°C with 5% $CO_2$ and a fully humidified atmosphere) for 5 or 10 days After incubation, the cell suspensions were washed three times with glucose-phosphate buffer to remove all extracellular Ara-C. The remaining cell pellet was resuspended in FCS and used for clonogenic assay.

**Clonogenic assay** After 5 and 10 days of exposure to Ara-C, the surviving numbers of CFU-L and CFU-GM were assessed in a semisolid assay. The conditions needed for CFU-L and CFU-GM are identical.[19] Cells suspensions were prepared in Iscove's medium supple-

mented with 20% FCS, 0.3% (w/v) Bacto-Agar (Difco, Detroit, Michigan), 5 U/ml IL-3, 250 U/ml GM-CSF, and 250 U/ml G-CSF, and duplicates were cultured in 35- x 10-mm culture dishes (Costar, Cambridge, Massachusetts) at 37°C in a fully humidified atmosphere containing 5% $CO_2$. The cell concentrations were adapted to previously determined plating efficiencies in order to obtain an adequate number of 100-200 aggregates per plate After 10 days the total number of clusters (5-40 cells) and colonies (>40 cells) was counted. A CFU-L aggregate usually consisted of <60 immature cells, whereas a CFU-GM aggregate mostly contained >200 mature granulocytes and/or macrophages.[20] To evaluate the control survival of clonogenic cells after 5 or 10 days of liquid incubation, cells were also immediately plated in agar, that is, without preincubation.

**Curve fitting** The Ara-C dose-response curves were fitted with the following formula:

$$f(x)^2 = \frac{a}{1+(bx)^c}$$

In this formula $a$ stands for the maximal plating efficiency (100%, measured when the cells are incubated without Ara-C) and $b$ for the Ara-C concentration at which inhibition is 70%; $c$ represents the slope of the curve. The model was fitted according to the Gauss-Newton regression procedure with the use of the least squares criteria.[21] The Ara-C concentrations resulting in 50% inhibition of clonogenicity ($ID_{50}$) were calculated from these curves.

**Statistics** The data are represented as the mean ± SD. Group data were compared by Student's t-test.

## Results

### Survival of clonogenic cells during liquid incubation

Before the liquid incubation test system was employed, we checked whether sufficient numbers of normal and leukemic clonogenic cells would be present even after incubation periods as long as 10 days. Table 1 presents the number of clonogenic cells cultured in agar after 5- or 10-day liquid incubation in the presence of IL-3, GM-CSF, and G-CSF, compared to the number seen after immediate plating in agar. The number of leukemic aggregates cultured without preincubation varied considerably from 76 to 30,373 per 2 x $10^5$ plated cells. The normal bone marrow progenitor cells showed a smaller range: 121-327 aggregates per 2 x $10^5$ cells. After 5-day incubation, 25-51,125 aggregates per 2 x $10^5$ leukemic cells and 166-863 per 2 x $10^5$ normal bone marrow cells were counted. When the number of aggregates cultured without preincubation was considered 100%, the percentage of clonogenic cells pre-

**Table 1** Survival of clonogenic cells after liquid incubation without Ara-C in the presence of IL-3 plus GM-CSF plus G-CSF

| | | Duration of incubation[a] | | |
|---|---|---|---|---|
| Number | Cell type | 0 Days | 5 Days | 10 Days |
| 1 | M2[b] | 23,400[c] | 34,600[c] (148)[d] | 16,383[c] (70)[d] |
| 2 | M2 | 195 | 1227 (629) | 185 (95) |
| 3 | M2 | 14,400 | 6020 (42) | 2863 (20) |
| 4 | M2 | 5298 | 2229 (42) | 1172 (22) |
| 5 | M2 | 76 | 25 (33) | 15 (20) |
| 6 | M4 | 30,373 | 51,125 (168) | 37,325 (123) |
| 7 | M4 | 2840 | 18,475 (651) | 17,760 (625) |
| 8 | M4 | 95 | 151 (159) | 32 (34) |
| 9 | M5 | 1448 | 7535 (520) | 947 (65) |
| 10 | Normal | 327 | 289 (88) | 89 (27) |
| 11 | Normal | 318 | 863 (271) | 905 (284) |
| 12 | Normal | 129 | 166 (129) | 77 (59) |
| 13 | Normal | 121 | 621 (513) | 313 (259) |

a Cells were plated in agar either directly (= incubation period of 0 days) or after an incubation period of 5 or 10 days.

b Leukemic marrow classified according to FAB classification

c Absolute number of aggregates (>5 cells) per $2 \times 10^5$ cells.

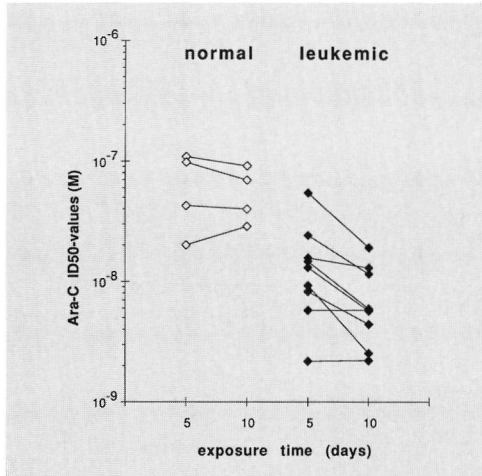
d Percentage of aggregates present after 5- or 10-day incubation considering the number of aggregates cultured without incubation to be 100%

sent after 5-day incubation was 33%-651% for the leukemic samples and 88%-513% for the normal bone marrows. After 10-day incubation, these percentages were 20%-625% for the leukemic cells and 27%-284% for the normal bone marrow cells. Except one normal bone marrow (no. 11), all samples contained lower numbers of clonogenic cells after 10-day liquid incubation compared to 5-day incubation. The survival of leukemic and normal bone marrow clonogenic cells during liquid incubation followed different patterns. Some samples contained more clonogenic cells after 5- and 10-day incubation than without incubation (nos. 6, 7, 11, and 13). In other cell samples (nos. 1, 2, 8, 9, and 12), the number of clonogenic cells after 5-day incubation was higher and after 10-day incubation lower than the number cultured after immediate plating in agar In sample nos. 3, 4, 5, and 10 the number of clonogenic cells decreased with the extension of the incubation period.

### Exposure to Ara-C for 5 days

Five-day exposure to Ara-C in the presence of IL-3, GM-CSF, and G-CSF inhibited the colony growth of leukemic as well as normal bone marrow cells. The mean Ara-C dose resulting in 50% inhibition ($ID_{50}$) of the CFU-L was 1.64 ±

**Figure 1** The concentrations of Ara-C (x $10^{-8}$ M) resulting in 50% inhibition of the survival of clonogenic normal bone marrow cells ($n = 4$) and leukemic blasts ($n = 9$) exposed to Ara-C for 5 and 10 days in the presence of IL-3, GM-CSF, and G-CSF.



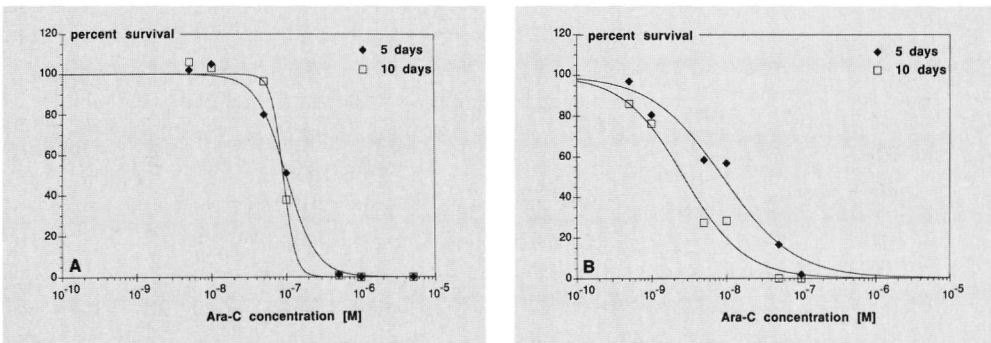




**Figure 2** Representative examples of Ara-C dose survival curves. The survival percentages are expressed as a percentage of two duplicate control cultures. The curves in the left part of the figure (2a) were obtained with normal bone marrow cells, whereas the curves in the right part of the figure (2b) were obtained with leukemic blasts. The cells were exposed to various concentrations of Ara-C for 5 or 10 days in the presence of IL-3, GM-CSF, and G-CSF.

1.56 x $10^{-8}$ M (n = 9), whereas the mean $ID_{50}$ value for the normal bone marrow cells was 6.74 ± 4.29 x $10^{-8}$ M Ara-C (n = 4). Seven of the leukemic samples were more sensitive to Ara-C cytotoxicity than the normal bone marrows (Fig. 1). Although the leukemic cells tended to be more sensitive to Ara-C than the normal cells, the difference was not significant ($p = 0.096$; Table 2).

## Effect of prolongation of the Ara-C exposure to 10 days

In order to enable more clonogenic cells to traverse the cell cycle and thereby make them more sensitive to Ara-C, the exposure time was extended from 5 to 10 days. Three out of four normal bone marrow samples showed a slight increase in Ara-C sensitivity, whereas one became less sensitive (Fig. 1). Overall, however, the cytotoxic effect towards CFU-GM remained about the same (mean $ID_{50}$ values after 5-day exposure, 6.74 ± 4.29 x $10^{-8}$ M, com-

**Table 2** Relation between Ara-C exposure time and cytotoxicity

| Exposure time (days) | CFU-GM | *n* | CFU-L | *n* | *p*[a] |
|---|---|---|---|---|---|
| 5 | 6.74 ± 4.29[b] | 4 | 1.64± 1.56 | 9 | 0.096 |
| 10 | 5.72 ± 2.83 | 4 | 0.78± 0.56 | 9 | 0.039[c] |

a *p*-value obtained with Student's t-test.
b Mean Ara-C concentration (± SD) x $10^{-8}$ M that inhibited 50% of the normal bone marrow (CFU-GM) and leukemic (CFU-L) clonogenic cells.
c Significant.

pared to 5.72 ± 2.83 x $10^{-8}$ M after 10-day exposure; Table 2). In Figure 2A, representative inhibition curves of normal CFU-GM are plotted, showing an equal sensitivity to Ara-C regardless of whether they were exposed to Ara-C for 5 or 10 days.

The sensitivity of leukemic blasts to Ara-C increased substantially in seven out of nine patients when the exposure time was prolonged from 5 to 10 days. The remaining two leukemic samples showed no change in cytotoxicity (Fig. 1). For the leukemic samples the mean $ID_{50}$ value decreased from 1.64 ± 1.56 x $10^{-8}$ M after 5-day exposure to 0.78 ± 0.56 x $10^{-8}$ M after 10-day exposure (Table 2). This mean $ID_{50}$ value of the leukemic samples after 10-day exposure was significantly different from the $ID_{50}$ value of the normal samples ($p = 0.039$; Table 2). As demonstrated in Figure 1, all leukemic samples became more sensitive than the normal progenitor cells after 10-day exposure to Ara-C. This change in sensitivity of the leukemic versus the normal samples is illustrated in the inhibition curves shown in Figure 2.

## Discussion

To investigate the effect of Ara-C on leukemic cell populations *in vitro*, we prefer to evaluate the toxicity of this drug on clonogenic cells, because clonogenic cells (especially the stem cells, which possess self-renewal capacity) are responsible for the maintenance of the leukemia.[1,2] Ara-C is preferentially cytotoxic to proliferating cells.[4,5] This means that short-term exposure to Ara-C will exhibit little or no effect on the non- and very slowly cycling proportion of clonogenic cells.[10] This kinetic resistance may be overcome by prolongation of the Ara-C exposure time, because per time unit a certain number of clonogenic cells are triggered into proliferation cycle.[1] Kinetic resistance also may be less when cells are stimulated to proliferate by CSF(s).

Studies performed by other investigators indicated that selected leukemic cells cultured with crude CSF in suspension for up to 238 days still contained clonogenic cells able to form colonies morphologically identical to the colonies cultured at the beginning of the incubtion.[22,23] We stimulated normal bone marrow and leukemic cells optimally with the combination of IL-3,

GM-CSF, and G-CSF for 5 and 10 days in suspension and subsequently assessed the survival of the clonogenic cells. To minimize the influence of endogenously produced CSF in this study, leukemic samples with spontaneous colony growth were excluded. Both increase and decrease in number of aggregates were observed during 10 days of incubation (Table 1), confirming earlier observations.[22] These patterns could be a reflection of the real growth potential or could have been caused by the culture conditions Because the suspension cultures were started with low cell concentrations and the cell numbers after 5 and 10 days of culture were between 50% and 200% of the initial cell numbers (data not shown), overcrowding was not the case. A relation between the patterns and the toxic effect of Ara-C was not found. Sufficient numbers of colony-forming cells were present even after 10 days of incubation (Table 1). Therefore, the test system was considered appropriate to investigate whether prolongation of the Ara-C exposure time to 10 days would result in an increased cytotoxicity towards leukemic clonogenic cells and normal progenitor cells.

When leukemic and normal clonogenic cells were exposed to Ara-C for 5 days in the presence of IL-3, GM-CSF, and G-CSF, in most cases the inhibition of CFU-L was higher than the inhibition of CFU-GM, but the difference was not significant (Table 2). These results were comparable with the preliminary data using HPCM instead of recombinant growth factors.[11] Prolongation of the Ara-C exposure time to 10 days resulted in an increased cytotoxicity towards most of the leukemic samples, but not towards the normal bone marrow cells This implied that exposure to Ara-C for 10 days resulted in a more preferential kill of leukemic clonogenic cells. Two of the nine investigated leukemias did not show an increased inhibition when they were exposed to Ara-C for 10 days instead of 5 days. It is striking that precisely these two leukemias were the most sensitive to the toxic effect of 5-day Ara-C exposure and were already inhibited to a much greater extent than the normal clonogenic cells.

Because the cytotoxic effect of Ara-C can be considered as a reflection of the proliferative activity, our observation would indicate that normal progenitor cells proliferated less than leukemic progenitor cells. An earlier report, in which the proliferative state of CFU-L and CFU-GM was measured by tritiated thymidine ($^3$H-TdR) labeling, indeed showed that the labeling index was higher for the leukemic progenitors.[7] An alternative explanation for the difference in cytotoxicity between the normal and the leukemic progenitors could be that there were differences in the levels of cytidine kinase and cytidine deaminase, thus altering the effective dose of Ara-C to which they were exposed. However, in previous experiments the Ara-C fraction left in the medium after 5 days of exposure to $10^{-6}$ M Ara-C was a mean of 52% for normal CFU-GM and 41% for CFU-L, whereas no relation was found between the residual Ara-C concentration and the $ID_{50}$ values (unpublished observations).

Because selected leukemic samples were used that formed aggregates only when they were stimulated with CSF, we have not proven yet whether

the same differential kill applies for *in vitro* spontaneously growing leukemias or leukemias not forming aggregates *in vitro*. Obviously, the nongrowing leukemias cannot be tested in this clonogenic assay system, whereas the effect on spontaneously growing leukemias is under current investigation.

Recently, selective kill of leukemic myeloblasts versus normal CFU-GM after only 96-h exposure to Ara-C was reported in a study investigating the possibilities for *ex vivo* bone marrow purging.[24] However, in that study the bone marrow was exposed to Ara-C in the presence of deoxycytidine, which preferentially protects normal CFU-GM from the toxicity of Ara-C.[25] Moreover, the cells were exposed to Ara-C in a culture medium lacking CSF. This unfavorable condition also contributed to the observed selective kill because after 96-h incubation the control growth of CFU-L dropped to 28%, whereas normal CFU-GM control growth remained 100%.

The cells cultured after 10 days of liquid incubation can be considered as clonogenic cells with self-renewal capacity.[22,23,26] Those cells and their susceptibility to Ara-C play an important role in the clinical outcome of antileukemic treatment. Patients with low *in vitro* numbers of blast progenitors with self-renewal capacity had a significantly greater chance of a successful remission induction.[27] Moreover, in a study where leukemic blasts in suspension were exposed to Ara-C for 7 days and replated in methylcellulose to assess the surviving clonogenic cells, a significant relation between Ara-C $ID_{90}$ values and a successful remission induction was found.[23]

It has been reported that the balance between self-renewal and terminal division can be influenced by culturing leukemic cells in the presence of IL-3, GM-CSF, or G-CSF alone.[27] The responses varied for the different tested leukemias, and the influence on this balance in normal bone marrow was not described. When leukemic cells were cultured in the presence of a particular growth factor that favored for self-renewal, an increased sensitivity to Ara-C was noticed.[28] An alternative to the optimal stimulation of all leukemic clonogenic cells with the combination of IL-3, GM-CSF, and G-CSF may be to determine for individual leukemias the single or the combination of recombinant growth factor(s) that more selectively stimulates the leukemic stem cells. Growth factors with relatively little stimulatory effect on normal stem cells are of particular interest. In the near future, leukemic patients may be treated with a therapy consisting of these individually determined growth factors combined with low dosages of Ara-C (or other cell cycle-specific agents). The dosages of Ara-C needed in this study for an effective kill were in the same range as the plasma concentrations reached with *in vivo* low-dose Ara-C administration.[29] Low-dose Ara-C therapy can be given to outpatients and usually causes only minimal extramedullary toxicity. This treatment schedule has already been applied in patients with myelodysplastic syndromes who received low-dose Ara-C in combination with GM-CSF.[30]

A theoretical application of this treatment modality could be *ex vivo* purging of bone marrow with the combination of Ara-C and growth factors. Because this implies a 10-day culture of the bone marrow, a major concern would be the survival of the hematopoietic stem cells in this liquid culture system (in contrast to the 'natural' environment of the patient). As an alterna-

tive, combined Ara-C and growth factor exposure in a long-term bone marrow culture (LTBMC) system could be considered. This system has proven to retain repopulating ability for at least 11 days,[31] and the system by itself seems to favor the maintenance of normal hematopoiesis [32]

In short, our results show that by prolonged exposure to low-dose Ara-C in the presence of the hematopoietic growth factors IL-3, GM-CSF, and G-CSF, more leukemic than normal bone marrow clonogenic cells with self-renewal capacity are killed.

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# Chapter 3

***In vitro response of blasts to IL-3, GM-CSF, and G-CSF is different for individual AML patients: factors that stimulate leukemic clonogenic cells also enhance ara-C cytotoxicity***

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## Summary

In vivo, growth factors are currently investigated for their capacity to trigger leukemic stem cells into cycle and thus overcome kinetic drug resistance. In this study, the susceptibility of leukemic clonogenic cells to individual growth factors was related to cytosine-arabinoside (Ara-C) sensitivity The effects of interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and combinations of these recombinant hematopoietic factors were tested on blast cells of nine acute myeloid leukemia (AML) patients. Growth factor responses were assessed in semi-solid clonogenic assay and in a 10-day liquid culture followed by clonogenic assay. Heterogeneity in growth factor response was observed in both test systems, resulting in a variable pattern for individual leukemias. In the majority of cases (six of nine) the response patterns in the semi-solid and liquid cultures were divergent. To test the Ara-C sensitivity, leukemic blasts were exposed in liquid to various concentrations of Ara-C in the absence and presence of preselected growth factors. After 10 days, the number of surviving leukemic colony-forming cells (CFU-L) was assessed. Exposure to Ara-C in the presence of optimal stimulatory factor(s) resulted in a 3- to 1000-fold increase of the Ara-C toxicity in seven patients. The Ara-C concentrations resulting in 50% inhibition of clonogenicity ($ID_{50}$) were 0.48-123 x $10^{-8}$ M Ara-C in the absence of stimulatory growth factors, versus only 0.12-0.40 x $10^{-8}$ M Ara-C in the presence of these factors. In two patients, addition of one or more factors neither increased the number of CFU-L in liquid nor enhanced the Ara-C toxicity. Even in the absence of growth factors the $ID_{50}$ values in these cases were as low as 0.20 and 0.28 x $10^{-8}$ M Ara-C and in the same range as the $ID_{50}$ values observed with maximum growth factor stimulation in the other seven patients. These results indicate that Ara-C cytotoxicity can be enhanced by individually selected, clonogenic cell growth-promoting hematopoietic factors.

## Introduction

A substantial number of leukemic myeloblasts are non- or very slowly cycling.[1-4] These cells may escape antileukemic treatment with cell cycle-specific drugs like cytosine-arabinoside (Ara-C). This phenomenon, referred to as kinetic resistance, is considered to be an important mechanism for Ara-C resistance in acute myeloid leukemia (AML) and can be distinguished from metabolic resistance.[5] Previous *in vitro* studies showed that prolongation of the Ara-C exposure time up to 10 days substantially increased the toxicity of Ara-C towards leukemic clonogenic cells [6,7] During prolonged exposure, temporarily noncycling cells were allowed to enter the proliferation cycle and slowly cycling cells apparently became more sensitive to Ara-C.[8,9] By this design, kinetic resistance was at least partially overcome. An additional option for circumventing kinetic resistance could be proliferation induction. Multiple studies show that both *in vitro* and *in vivo* the growth of myeloid leukemic blasts is regulated by the same colony-stimulating factors that control normal hematopoiesis.[10-24] Particularly interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) can stimulate the proliferation of leukemic myeloblasts.[12,15,17,20,22,24] Therefore, we investigated whether stimulation of leukemic clonogenic cells with these growth factors could overcome the kinetic resistance to Ara-C. For individual leukemias the response patterns to IL-3, GM-CSF, G-CSF, and combinations of these factors were determined. Factor responsiveness was tested in a semi-solid system and in a liquid culture, followed by clonogenic assay. Since previous experiments showed that the toxicity of clinically relevant Ara-C dosages was tested most optimally by a 10-day Ara-C exposure,[6,7] this test system was applied to evaluate the toxic effect of the combined Ara-C and growth factor treatment.

Our results show that the kinetic resistance of leukemic clonogenic cells to Ara-C can be decreased by stimulation with individually adapted hematopoietic growth factors.

## Materials and Methods

**Patients** Bone marrow samples from nine previously untreated AML patients (five females, four males) were tested. The median patient age was 53 years (range 16-66). The diagnosis according to the French-American-British (FAB) classification was M2 (n = 5), M4 (n = 2), M5 (n = 1) and M6 (n = 1).[25] The samples contained median 68% blasts (range 28-94). Normal bone marrow was obtained from five patients undergoing cardiac surgery. Informed consent was given in all cases.

**Bone marrow collection, cryopreservation, thawing** Bone marrow was collected in sterile buffered acid-citrate dextrose (pH=7.0). The majority of the red blood cells and mature myeloid cells were removed by centrifugation on Ficoll (1.085 g/ml, Sigma, St. Louis, Missouri, USA). The cells were cryopre-

served in 1-ml vials in liquid nitrogen with a temperature-controlled freezer (Kryo 10; Planer Biomed, Sunbury, Middlesex, UK). Vials contained 5-20 x $10^6$ interphase cells suspended in Iscove's medium (Flow Laboratories, Irvine, Scotland), supplemented with 5% fetal calf serum (FCS; HyClone, Logan, Utah, USA) and 10% dimethylsulfoxide (DMSO). Just prior to the experiment, cells were thawed rapidly in a 37°C waterbath resulting in a cell recovery of >90%. The detailed procedure of freezing and thawing has been described before.[26]

**Human recombinant growth factors** Human recombinant IL-3 was kindly donated by Sandoz BV (Uden, The Netherlands) Human recombinant GM-CSF and G-CSF, were a kind gift from Behring (Marburg, Germany). Single factors and combinations were added to both the semi-solid cultures and the liquid cultures at final concentrations of 40 ng/ml, 5 ng/ml, and 5 ng/ml respectively. These concentrations resulted in plateau stimulation.

**Clonogenic assay (CFU)** Cell suspensions were prepared in Iscove's medium supplemented with 20% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin (Flow Laboratories), and 0.3% (w/v) bacto-agar (Difco, Detroit, Michigan, USA). To determine the effects of recombinant growth factors on leukemic and normal colony-forming cells (CFU-L/GM), no factor, the single factors and combinations of two or three factors were added to the semi-solid assay. All samples were exposed to the same panel of growth factors. Duplicates were cultured in 35- x 10-mm culture dishes (Costar, Cambridge, Massachusetts, USA) at 37°C in a fully humidified atmosphere containing 5% $CO_2$. In order to obtain an adequate number of about 200 aggregates/dish, 2 x $10^3$-2 x $10^5$ cells were plated After 7-10 days, clusters (5-40 cells) and colonies (>40 cells) were counted. The leukemic samples all had a high plating efficiency and the uniformly appearing CFU-L aggregates consisited of <60 immature cells. Compared to the leukemic samples, the plating efficiency of the normal BM samples was lower, whereas the CFU-GM aggregates usually contained >200 mature granulocytes and/or macrophages.

**Liquid culture** Maximally $10^5$ cells/ml were suspended in tubes (Falcon, Becton Dickinson, Lincoln Park, New Jersey, USA) containing Iscove's medium supplemented with 20% FCS, 50 IU/ml penicillin, and 50 µg/ml streptomycin. Stimulation by IL-3, GM-CSF, and G-CSF was tested by adding no factor, the single factors, and combinations of two or three factors to the medium. All samples were exposed to the same panel of growth factors. The cell suspensions were cultured in an incubator (37°C, 5% $CO_2$ in air and fully humidified atmosphere) for a period of 10 days. Thereafter, cells were washed with glucose-phosphate buffer and the number of CFU was assessed by clonogenic assay stimulated with the combination of IL-3, GM-CSF, and G-CSF (see clonogenic assay).

**Ara-C exposure** Leukemic cells were exposed to Ara-C (Upjohn, Kalamazoo, Michigan, USA) during a 10-day liquid culture with or without growth factor(s). The final added concentrations varied from $10^{-10}$ to $5 \times 10^{-5}$ M Ara-C. Dilutions were prepared from freshly thawed Ara-C stock solutions. The drug was checked for concentration, purity, and stability by high-performance liquid chromatography (HPLC).[27] After the exposure, cells were washed three times to remove all nonphosphorylated cellular and extracellular Ara-C. Subsequently, the number of surviving CFU-L was determined by clonogenic assay in the presence of the combination of IL-3, GM-CSF, and G-CSF (see above) In nine experiments, after the 10-day exposures, the remaining Ara-C concentrations in the suspensions were determined by HPLC.

**Curve fitting and statistics** To fit the Ara-C dose response curves, the following formula was used:

$$f(x)^2 = \frac{a}{1+(bx)^c}$$

In this formula $a$ stands for the maximal plating efficiency (100%, measured when the cells are incubated without Ara-C) and $b$ for the Ara-C concentration at which inhibition is 70%; $c$ represents the slope of the curve. The data were fitted according to the Gauss-Newton regression procedure with the use of the least squares criteria.[28] The Ara-C concentrations resulting in 50% inhibition of clonogenicity ($ID_{50}$) were calculated from these curves.

## Results

### Growth responses in semi-solid cultures

To assess the responses of leukemic clonogenic cells to growth factors, blasts from nine AML patients were cultured in semi-solid assays both in the absence and presence of IL-3, GM-CSF, G-CSF, and combinations of these factors. The mean number of cultured aggregates per $2 \times 10^5$ plated cells is depicted in Figure 1. In all cases, the cluster-to-colony ratio exceeded 5. Mostly, a pattern of large (maximum cluster size, 40 immature cells) or small (maximum cluster size, 20 immature cells) clusters was observed. Spontaneous growth in the absence of exogenous growth factors was observed in three cases (nos. 1, 4, 5). In five cases (nos. 1, 2, 6, 7, 8) G-CSF was the best single stimulator. In the other four cases similar responses to either single factor (nos. 3, 4) or an almost equal stimulation with two single factors and a lower aggregate number when cultured with the third single factor (nos. 5, 9) was found. In general, the number of aggregates increased when more than one recombinant factor was added. Synergism between two and/or three factors was observed in seven cases, for example between IL-3/G-CSF, GM-CSF/G-CSF, and IL-3/GM-CSF/G-CSF in case 6.

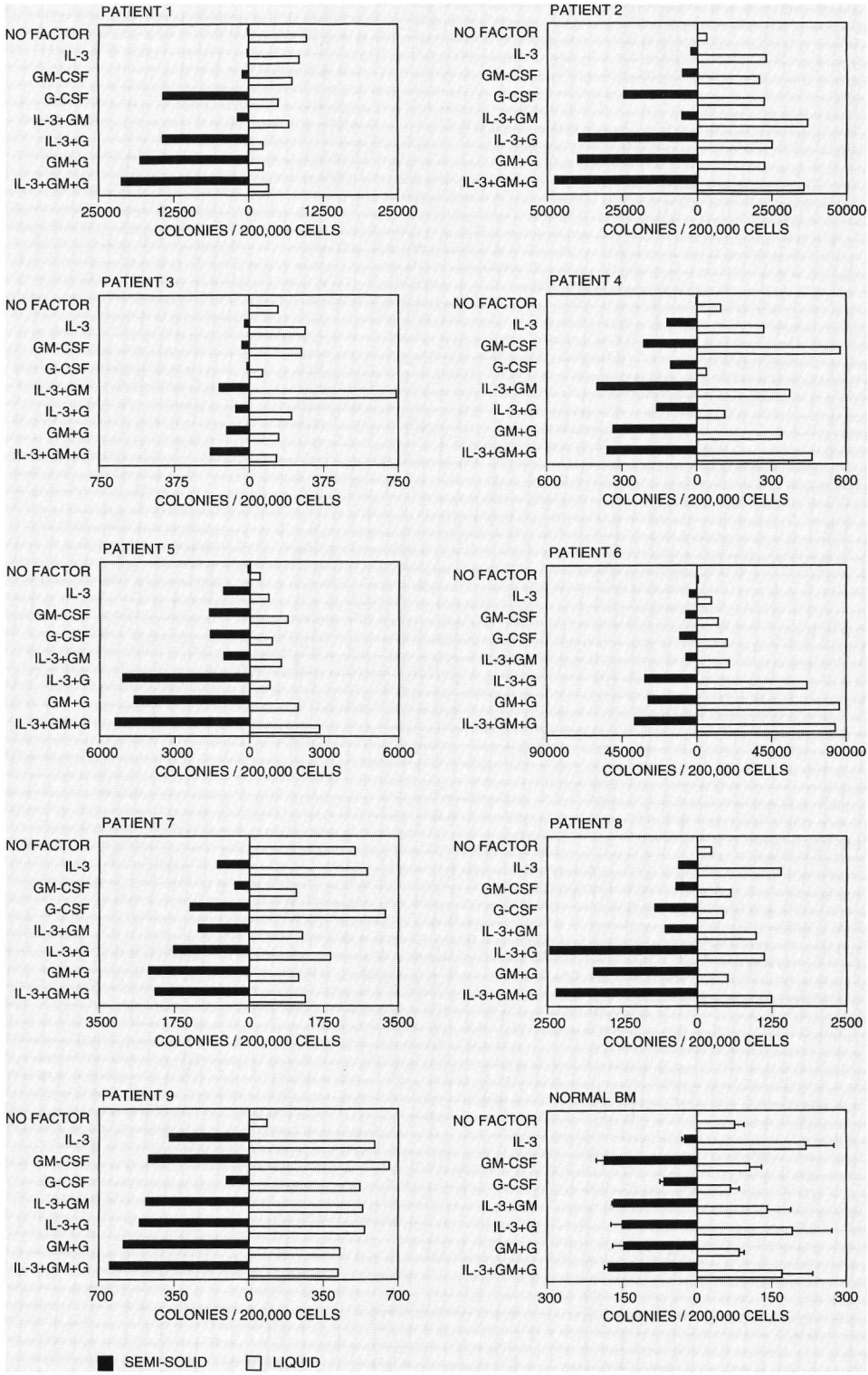

PATIENT 1
NO FACTOR
IL-3
GM-CSF
G-CSF
IL-3+GM
IL-3+G
GM+G
IL-3+GM+G
25000 12500 0 12500 25000
COLONIES / 200,000 CELLS
PATIENT 2
50000 25000 0 25000 50000
COLONIES / 200,000 CELLS
PATIENT 3
750 375 0 375 750
COLONIES / 200,000 CELLS
PATIENT 4
600 300 0 300 600
COLONIES / 200,000 CELLS
PATIENT 5
6000 3000 0 3000 6000
COLONIES / 200,000 CELLS
PATIENT 6
90000 45000 0 45000 90000
COLONIES / 200,000 CELLS
PATIENT 7
3500 1750 0 1750 3500
COLONIES / 200,000 CELLS
PATIENT 8
2500 1250 0 1250 2500
COLONIES / 200,000 CELLS
PATIENT 9
700 350 0 350 700
COLONIES / 200,000 CELLS
NORMAL BM
300 150 0 150 300
COLONIES / 200,000 CELLS
SEMI-SOLID
LIQUID

**Figure 1** Of nine AML patients and five normal bone marrow donors, the growth factor responses of blasts and normal bone marrow cells were determined in semi-solid and liquid cultures. In the semi-solid culture cells were exposed to: no factor, IL-3, GM-CSF, G-CSF, and combinations of these factors In the liquid cultures cells were exposed to the same panel of growth factors for 10 days followed by semi-solid assay stimulated with the combination of IL-3, GM-CSF, and G-CSF The responses are expressed as the number of CFU-L/GM per 2 x $10^5$ 'input' cells. The *left part* of each diagram represents the data obtained in semi-solid cultures The *right part* of each diagram shows the results obtained in the liquid cultures Notice that the absolute number of CFU substantially differs between individual patients. Response patterns of individual AML cases are presented in separate diagrams. Results obtained with normal bone marrow cells are combined and expressed as mean (±SEM) in one diagram

## Growth responses in liquid cultures

The growth factor responses in the liquid cultures are shown for each individual patient in Figure 1. In all cases, aggregates were detectable in the semi-solid assay after 10-day liquid culture without colony stimulating factor (CSF). Incubation with a single factor showed in one case (no. 8) that IL-3 was the most effective stimulator, in two cases GM-CSF (nos. 4 and 5), and in one case G-CSF (no. 6). Similar responses to all three single factors or almost equal results with two single factors and lower aggregate numbers in the presence of the third single factor were seen in the other five cases. In contrast to the responses in the agar cultures, addition of more than one growth factor did not tend to increase the number of colony-forming cells. Evident synergism between two and/or three factors could only be observed in case 6. The number of aggregates obtained by maximum stimulation during liquid culture was 139 ± 72% (mean ± 95% confidence interval) of the number of CFU-L obtained when cells were directly cultured in semi-solid.

Comparison of the response patterns of individual patients revealed that in six of the nine cases the growth responses in semi-solid and liquid culture were divergent Either the single factor which best supported the clonogenic cell growth had changed (as in case 8, where G-CSF gave the best stimulus in the semi-solid assay and IL-3 the best during liquid culture) or prominent differences had disappeared (for example case 2, in which IL-3, GM-CSF, and G-CSF revealed similar numbers after liquid stimulation, whereas the aggregate numbers in the primary culture were quite different).

## Enhancement of Ara-C toxicity by stimulation with growth factors

To test our hypothesis that proliferation induction enhanced cytotoxicity, the leukemic blasts were exposed to Ara-C for 10 days in the absence and presence of preselected factors (Table 1). Factors, which caused the most pronounced differences in stimulation during the liquid cultures, were preferentially chosen.

**Table 1** Enhancement of Ara-C toxicity by proliferation induction

| Patient no. | Added factor [a] | Control growth[b] | $ID_{50}$ value [c] |
|---|---|---|---|
| 1 | No factor | 9720 | 0.28 |
| | IL-3 | 8480 | 0.16 |
| | IL-3+G-CSF | 2400 | 0.36 |
| 2 | No factor | 3080 | 17.6 |
| | GM-CSF | 20600 | 0.40 |
| 3 | G-CSF | 70 | 0.47 |
| | IL-3+GM-CSF | 740 | 0.16 |
| 4 | G-CSF | 40 | 1.31 |
| | GM-CSF | 580 | 0.23 |
| 5 | No factor | 400 | 58.9 |
| | G-CSF | 900 | 2.54 |
| | IL-3+GM-CSF+G-CSF | 2810 | 0.32 |
| 6 | No factor | 650 | 123 |
| | IL-3 | 8750 | 0.26 |
| | GM-CSF+G-CSF | 86000 | 0.12 |
| 7 | No factor | 2480 | 0.20 |
| | GM-CSF | 1100 | 0.11 |
| | G-CSF | 3190 | 0.07 |
| 8 | No factor | 230 | 0.48 |
| | IL-3+GM-CSF+G-CSF | 1240 | 0.28 |
| | IL-3 | 1400 | 0.13 |
| 9 | No factor | 80 | 3.22 |
| | GM-CSF | 660 | 0.33 |

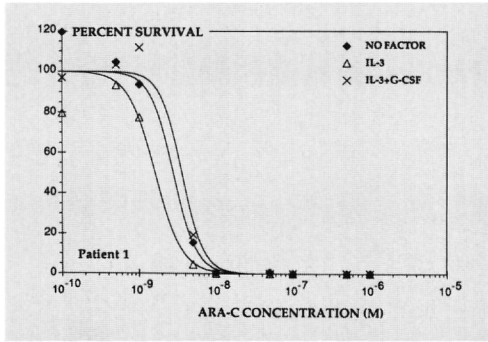
a Growth factor(s) added to the liquid culture.

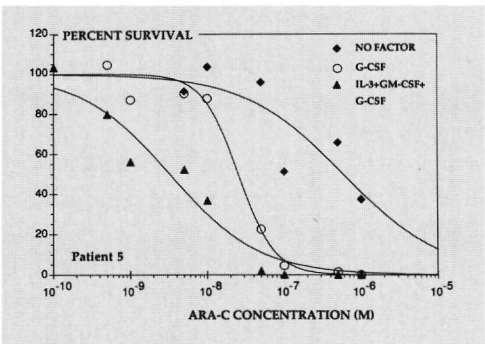
b CFU-L number per $2 \times 10^5$ 'input' cells, assessed in duplicate after 10-day liquid culture in the absence of Ara-C.

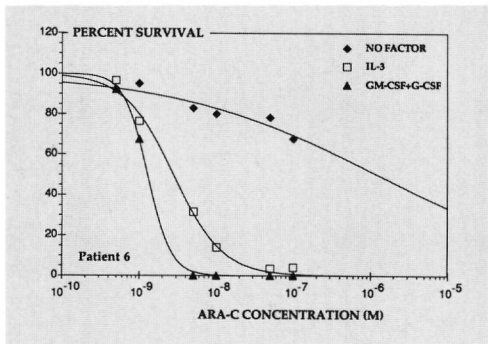
c Ara-C concentration $\times 10^{-8}$ M, that inhibited 50% of the CFU-L growth. The values were calculated from survival curves constructed after 10-day exposure to various concentrations of Ara-C.

In seven cases (nos. 2, 3, 4, 5, 6, 8, 9) growth factor(s) stimulated the leukemic clonogenic cells in liquid and a substantial increase of Ara-C toxicity was observed. In individual cases, better stimulation correlated with higher Ara-C sensitivity. The Ara-C dose survival curves of two representative examples (nos. 5 and 6) are shown in Figure 2. In case 5, G-CSF enhanced Ara-C toxicity. The $ID_{50}$ value was $2.54 \times 10^{-8}$ M Ara-C com-pared to $58.9 \times 10^{-8}$ M Ara-C in the absence of any factor. An even better stimulation with the combination of IL-3, GM-CSF, and G-CSF further increased the Ara-C sensitivity. The $ID_{50}$ value of $0.32 \times 10^{-8}$ M Ara-C was 180-fold lower than the $ID_{50}$ value observed in the absence of CSF. A similar increase in Ara-C sensitivity was observed in case 6. When stimulated with IL-3 alone and the combination of GM-CSF and

**Figure 2** Ara-C dose survival curves of patients 1, 5, and 6. For a period of 10 days, cells were exposed to various concentrations of Ara-C in the presence of the indicated growth factor(s). Subsequently, the number of surviving CFU-L was determined by clonogenic assay stimulated with the combination of IL-3, GM-CSF, and G-CSF. The CFU-L survival was expressed as a percentage of control cultures.




G-CSF respectively, the $ID_{50}$ value decreased from 123 x $10^{-8}$ M Ara-C in the absence of growth factor to 0.26 x $10^{-8}$ M Ara-C in the presence of IL-3 and 0.12 x $10^{-8}$ M Ara-C in the presence of GM-CSF and G-CSF. Thus, with this latter combination of factors, a 1000-fold enhancement of Ara-C toxicity was achieved. Overall, stimulation with recombinant growth factors resulted in a decrease of the median Ara-C $ID_{50}$ values from 3.22 (range 0.47-123) to 0.23 (range 0.12-0.40) x $10^{-8}$ M. In individual patients a 3- to 1000-fold increase of Ara-C toxicity was observed in the presence of growth factor(s).

In two cases (nos. 1 and 7) addition of growth factor(s) did not increase the number of CFU-L in liquid. In these cases growth factors could not enhance the Ara-C toxicity, as shown by the representative Ara-C survival curves of patient 1 (Fig. 2). Even in the absence of growth factors the $ID_{50}$ values of these two patients were as low as 0.28 and 0.20 x $10^{-8}$ M Ara-C respectively. These values were in the same range as the $ID_{50}$ values observed with maximum growth factor stimulation in the other seven patients.

## Ara-C concentration during 10-day liquid culture

Ara-C can be inactivated to uracil arabinoside (Ara-U),[29] particularly in suspensions with a relatively high cell concentration.[6] To minimize this deamination, cell concentrations of less than $10^5$/ml were used. With these relatively low cell concentrations no significant decrease in Ara-C concentration

could be detected at the end of the 10-day culture. In the suspensions to which initially $1 \times 10^{-6}$ M Ara-C was added, the remaining concentration was $0.96 \pm 0.11 \times 10^{-6}$ M Ara-C (mean ± 95% confidence interval, n = 9).

## Discussion

In the present study we investigated the potential role of IL-3, GM-CSF, and G-CSF to overcome the kinetic resistance of AML blasts to Ara-C. We focused our investigations on the clonogenic subpopulation of the leukemic blasts, since these clonogenic cells are held responsible for the maintenance of the leukemia.[8,9]

In nine leukemias the response to IL-3, GM-CSF, G-CSF, and combinations of these factors was investigated in semisolid culture. As already noticed by others,[12,17,20,24] a remarkable diversity in response was observed (Fig. 1) and no evident relationship between response pattern and FAB type was seen. Synergism between 2 or 3 factors was observed in the majority of cases, most often between G-CSF and one or two other factors. The response patterns in liquid cultures were also determined (Fig. 1). Heterogeneity in response was again observed. In the majority of cases (six of nine) the responses to growth factors in liquid cultures were divergent from those in the semisolid cultures. These data corroborate those of a previously reported study,[20] but contradict another.[24] It is possible that in the liquid cultures hierarchically more immature progenitor cells are cultured, compared to more mature progenitors in the semisolid culture.[20,30] These distinct progenitors may have different biological properties, which may explain the observed discrepancy. The marked variation in response to IL-3, GM-CSF, and G-CSF observed in leukemias is in contrast with the reported uniform response of normal hematopoietic progenitors.[11,12,16,19] We studied the growth factor response of normal myeloid-macrophage progenitor cells (CFU-GM) and found identical response patterns for five different normal bone marrows tested (Fig. 1). Surface-marker studies suggest that leukemic clonogenic cells originate at different maturation stages of hematopoiesis.[31] The heterogeneous response of leukemias, therefore, may reflect the position at which the leukemic cells are arrested.

Based on previous results,[6,7] the possible enhancement of Ara-C toxicity towards leukemic clonogenic cells by growth factors was studied during a 10-day Ara-C exposure experiment. The growth factors were co-incubated to achieve optimal stimulation during the whole drug exposure period. Since Ara-C inactivation is cell concentration dependent,[6] the cell concentration in the test system was intentionally lowered to less than $10^5$ cells/ml. Under this condition, on average only 4% of the Ara-C was deaminated during the 10-day liquid culture. In seven out of nine tested cases, growth factors increased the number of leukemic clonogenic cells during the liquid cultures when compared to unstimulated cultures. In all these cases, a more effective stimulation corresponded with an increase of Ara-C toxicity (see Table 1). Overall,

the $ID_{50}$ values were 3- to 1000-fold lower in case of optimal stimulation. In the other two cases, growth factors were not able to increase the clonogenicity during liquid culture, and the Ara-C toxicity was not enhanced (Table 1). In the absence of exogenously added growth factors, these two leukemias were already extremely sensitive to Ara-C. The $ID_{50}$ values were equal to the values observed after maximal stimulation in the other seven leukemias. One of these two leukemias showed spontaneous growth in the semi-solid culture, suggesting autocrine colony-stimulating factor production or autonomous proliferation. This may explain the high toxicity of Ara-C in the absence of exogenous colony-stimulating factors. In case of optimal stimulation, relatively low Ara-C concentrations were sufficient to eradicate the leukemic clonogenic cells in all nine tested leukemias. *In vivo*, these Ara-C levels can be reached in the plasma of patients treated with subcutaneously administered low-dose (20 mg/m$^2$/d) Ara-C.[32]

Cells may escape Ara-C toxicity by kinetic or metabolic resistance or a combination of both. These mechanisms are reported to be of clinical importance in AML.[4,5] Seven of the nine tested leukemic samples showed increased Ara-C cytotoxicity after stimulation with growth factors. We may conclude that in these leukemias kinetic resistance was the major reason these blasts were relatively insensitive to Ara-C toxicity. If metabolic resistance had been more important, no substantial increase in toxicity would have been observed after recruitment with growth factors.

Several other papers reported that growth factors can enhance the cytotoxic effect of cell cycle-specific agents like Ara-C and hydroxyurea towards leukemic cells.[33-38] In these studies, AML blasts were exposed to the drugs either in the presence of a growth factor or after prestimulation. In contrast to our study, usually only one factor was investigated and the tested growth factor was not selected on the basis of individual responsiveness. Therefore, the growth factors used in these studies may not have been the most optimal stimulus for each tested leukemia. Moreover, the drug exposure time was relatively short, ranging from 3 to 24 h. The Ara-C concentrations tested were relatively high, varying from $10^{-7}$ M to $10^{-3}$ M. Even with these pharmacologically very high Ara-C concentrations, which are usually not reached for prolonged periods in the clinical situation,[32] a complete inhibition of CFU-L was never obtained. This suggests that these incubation periods were too short to fully eradicate the leukemic clonogenic cells. These results corroborate our data from earlier experiments, showing that during relatively short exposure periods of 1 h, 24 h, or 5 days, concentrations of at least $10^{-5}$ M Ara-C were necessary to inhibit CFU-L growth completely.[6] In the study of Miyauchi et al. cells of two patients were exposed to Ara-C in the presence of individually adapted growth factors over a longer period (7 days).[38] However, the choice of the added factors was not based on the response in liquid, but on the shift from terminal division (methylcellulose) to self-renewal (suspension). No relation between the response in liquid (that is, an increase in CFU-L number) and the Ara-C sensitivity was found. Only when factors relatively favored self-renewal, a maximally twofold increased sensi-

tivity to Ara-C was observed. The calculated $ID_{90}$ values of 0.2-0.6 x $10^{-6}$ M Ara-C were about 10 times higher than observed with the 10-day exposure we applied.

The potential risk of the clinical application of growth factors may be proliferation induction of the normal hematopoietic stem cells. In combination with Ara-C the consequence could be an increased toxicity towards these cells resulting in prolonged hypoplasias. *In vitro* and *in vivo* data thus far suggest that in the presence of growth factors the toxicity of Ara-C towards normal progenitor cells is not higher than the toxicity observed with Ara-C alone.[37,39,40] We found that 10-day Ara-C exposure in the presence of the combination of IL-3, GM-CSF, and G-CSF resulted in a preferential kill of the leukemic versus the normal clonogenic cells.[7] Still, more studies in this direction need to be performed.

Several pilot studies have investigated the potential value of GM-CSF to enhance the cytotoxic efficacy of remission induction therapy in AML.[41] Considering the data presented, it seems less justified to treat all patients with the same factor, regardless whether their leukemia can be stimulated with that factor or not. Instead, it appears that growth factor(s) should be selected individually so that in AML patients recruitment of leukemic clonogenic cells can be obtained with optimal stimulatory factor(s). In about 80% of the AML cases, CFU-L can be cultured *in vitro*. This indicates that the majority of AML patients can be evaluated for their *in vitro* growth response. Further clinical studies have to prove whether the *in vitro* recruitment data reflect the *in vivo* biology.

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# Chapter 4

## *Detection of incorporated iododeoxyuridine in colonies by immunoperoxidase staining: a novel method to measure the proportion of cycling colony-forming cells*

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## Summary

In vitro suicide by tritiated thymidine ($^3$H-TdR), hydroxyurea (HU), or cytosine arabinoside (Ara-C) is assumed to reflect the proportion of colony-forming cells in S-phase at the time of exposure. However, these techniques are not always accurate. Nonradioactive iododeoxyuridine (IdUrd) is incorporated into DNA during S-phase and can be detected by monoclonal antibodies. In the present study, a new IdUrd application was developed to investigate the kinetics of hematopoietic progenitor cells. After incubation with IdUrd, colony-forming cells were cultured in semi-solid assay. An immunoperoxidase staining protocol was developed to detect IdUrd in cells of colonies in agar. Colony-forming cells in S-phase during the IdUrd exposure were postulated to give rise to IdUrd$^+$ colonies, whereas non-S-phase cells would generate IdUrd$^-$ colonies. Toxicity, sensitivity, and IdUrd inactivation studies indicated that progenitor cells could safely be pulse-labeled for 2 hours with 40 μM IdUrd, whereas prolonged labeling with 1 μM IdUrd was at least feasible for 5 days.

Molt-4 cells and normal bone marrow cells were used to compare IdUrd pulse-labeling with $^3$H-TdR suicide. Part of the Molt-4 cells were enriched for $G_1$- and S-phase cells by counterflow centrifugation. The bone marrow cells were either unstimulated or stimulated with growth factors. As a result, the accuracy of both techniques could be tested in populations with different quantities of S-phase cells. Wide confidence intervals of the suicide technique contrasted the small confidence intervals obtained with IdUrd pulse-labeling. For instance, the fraction of Molt-4 cells with 27.8% S-phase cells contained 17.7% (confidence interval -8.2 to 43.6%) clonogenic cells in S-phase when determined with $^3$H-TdR suicide. Of this fraction, the percentage of clonogenic cells in S-phase was 30.6% with a confidence interval of 25.5 to 36.2% when determined with IdUrd pulse-labeling. In our hands, the IdUrd pulse labeling was more accurate than the $^3$H-TdR suicide technique.

Thus far, kinetic studies of progenitors have been limited to the determination of the fraction of S-phase cells by suicide techniques. By prolonged IdUrd labeling, it is now possible to determine the proliferating fraction of progenitor cells.

## Introduction

Normal and leukemic progenitor cells can be cultured *in vitro* by semisolid assay.[1] This enables kinetic studies of these cells. Insight into the proliferative behaviour of progenitors will help to understand the biology of the hematopoietic system under physiologic and pathological conditions. Furthermore, therapeutic interventions, such as exposure to cytostatic drugs or growth factors, can be evaluated. The proliferative status of progenitors can be studied *in vitro* by suicide techniques.[2,3] After short-term exposure to $^{3}$H-TdR, HU, or Ara-C, the loss of progenitor cells is determined by colony-forming assay. The fractional reduction in cloning efficiency is considered to reflect the proportion of colony-forming cells in S-phase at the time of exposure. The estimation of progenitors in S-phase by suicide techniques may not always be accurate.[2,4,5] It has been reported that non-S-phase cells, probably late $G_1$ cells, can take up $^{3}$H-TdR and eventually will be killed.[4,5] Nevertheless, the commonly applied $^{3}$H-TdR suicide seems to be the most reliable of the available techniques.[4]

Previously, IdUrd has become available for kinetic studies. This nonradioactive thymidine analog is incorporated into DNA during S-phase and can be detected by anti-IdUrd antibodies.[6,7] Using flow cytometry (FCM) or immunocytochemistry, S-phase fractions of both normal and malignant cells have been determined [8-12]

To our knowledge, proliferation of hematopoietic progenitors has not been studied with IdUrd. The present study was designed to develop a new IdUrd technique to investigate the kinetic behaviour of colony-forming cells. During IdUrd exposure, only progenitor cells in S-phase will incorporate IdUrd into their DNA. Subsequently, when a colony-forming assay is performed, a single progenitor cell will form one colony. Cell division is expected to result in the distribution of parental DNA over the daughter cells. Therefore, detection of incorporated IdUrd in the cells of a particular colony will indicate that it originated from a colony-forming cell that has been in S-phase during the labeling period. Colony-forming cells not in S-phase during exposure will give rise to IdUrd$^{-}$ colonies. The fraction of progenitor cells in S-phase can be determined by pulse-labeling. The total proportion of cycling progenitors can be assessed after a prolonged labeling period.

Labeling of cells with 10 μM IdUrd for 30 minutes proved to be optimal for kinetic analysis by FCM.[8,9,13] With FCM, cells are analyzed immediately after labeling. As a result, dilution of the IdUrd signal due to cell division occurs infrequently. In contrast, IdUrd$^{+}$ colonies derive from labeled clonogenic cells that have divided several times to form a colony. To prevent that, the IdUrd signal will become undetectable, the concentration used to pulse-

label progenitors should be sufficiently high, and the labeling period as long as theoretically justified.

A 52% reduction in myeloid colonies was observed in a study in which normal bone marrow was incubated with 5 μM IdUrd for 24 hours.[14] IdUrd can be catabolized to 5-iodouracil (IUra) and further degradated.[14] Therefore, toxicity and IdUrd inactivation were considered to be major limitations for prolonged labeling.

In the present study, sensitivity, toxicity, and IdUrd inactivation were evaluated to determine the optimal concentration for pulse and prolonged labeling. A previously published protocol developed for immunological detection of IdUrd by FCM was improved and adjusted for application on agar cultures.[13] Comparative studies with $^{3}$H-TdR suicide were performed to establish the accuracy of IdUrd pulse-labeling.

It appeared that the fraction of colony-forming cells in S-phase can be determined more accurately with IdUrd pulse-labeling than with $^{3}$H-TdR suicide. Moreover, prolonged IdUrd labeling may create the ability to assess the fraction of proliferating progenitors.

## Materials and Methods

**Bone marrow collection, cryopreservation, and thawing** Normal bone marrow cells were obtained from healthy bone marrow donors. Informed consent was given in all cases. The cells were collected in sterile buffered acid-citrate dextrose (pH=7.0) and enriched for clonogenic cells by flotation centrifugation [15] In short, cells were mixed with Percoll (Pharmacia, Uppsala, Sweden) to obtain a density of 1.085 g/ml. From this suspension, 15 ml was layered under 30 ml of a 1.067 g/ml Percoll solution with 5 ml Hanks' balanced salt solution (HBSS) (Flow Laboratories, Irvine, Scotland) on top of it. After centrifugation, the cells with a density of $<1.067$ g/ml were collected and frozen in liquid nitrogen using a temperature-controlled freezer (Kryo 10; Planer Biomed, Sunbury, Middlesex, UK). Each vial contained $5 \times 10^{6}$ cells in Iscove's modified Dulbecco's medium (IMDM) (Flow Laboratories), supplemented with 10% heat inactivated fetal calf serum (FCS) (HyClone, Logan, UT), 50 IU/ml penicillin, 50 μg/ml streptomycin (both Flow Laboratories) and 10% dimethylsulfoxide (DMSO). Just prior to the experiments, cells were thawed in a 37°C waterbath, resulting in a recovery of >90%. Freezing and thawing procedures have been described in detail elsewhere.[16]

**Recombinant human growth factors** Recombinant human interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were a kind gift of Sandoz BV (Uden, The Netherlands). Recombinant human granulocyte colony-stimulating factor (G-CSF) was kindly donated by Behring (Marburg, Germany). Final concentrations of 40 ng/ml, 20 ng/ml, and 5 ng/ml of each, respectively, were used and resulted in plateau stimulation.

**Pulse and prolonged IdUrd labeling** Normal bone marrow cells ($10^4$ cells/ml) were suspended in IMDM supplemented with 20% FCS, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The cultures were either unstimulated or stimulated with the combination of IL-3, GM-CSF, and G-CSF. IdUrd (Sigma, St. Louis, MO) dilutions were prepared from freshly thawed stock solution. Based on the experience with FCM, pulse-labeling was performed for 2 hours at final concentrations of 5, 10, 20, 40, 60, and 80 μM IdUrd. In case of prolonged labeling, IdUrd at final concentrations of 0.5, 1, 2, 3, 4, 5, and 10 μM was added to the suspensions. Cells were labeled for a period of 1, 2, 3, 4, and 5 days. Labeling was performed at 37°C, in a fully humidified atmosphere containing 5% $CO_2$ in air. Subsequently, cells were washed three times with glucose-phosphate-buffered saline (G-PBS). The cell pellet was resuspended in FCS and used for clonogenic assay and cytocentrifugation. In each experiment, one sample not labeled with IdUrd was identically processed and served as negative control. The supernatants of the cell suspensions, incubated for 1 to 5 days with 1 μM IdUrd, were collected before washing and preserved at -20°C to determine the remaining IdUrd content by bioassay.

**Clonogenic assay (CFU-GM)** Cells were cultured in IMDM supplemented with 20% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 0.3% (w/v) bacto-agar (Difco, Detroit, MI). Cells were stimulated with the combination of IL-3, GM-CSF, and G-CSF. Duplicates were cultured in 35- x 10-mm culture dishes (Costar, Cambridge, MA) at 37°C in a fully humidified atmosphere containing 5% $CO_2$ in air. To obtain 200 to 400 aggregates per dish, 2 to 4 x $10^4$ cells in a total volume of 0.85 ml were plated. The cultures were ended after 5 to 7 days, when aggregates consisted of 20 to 60 cells. After counting the clusters (5 to 40 cells) and colonies (>40 cells), the agar cultures were dried by cytocentrifugation and used for immunoperoxidase staining.

**Cytocentrifugation of cells** Cells in suspension were sedimented onto a microscope slide by cytocentrifugation (Shandon, Pittsburgh, PA). Filter cards were moistened with 100 μl 5% human serum albumin (HSA) in PBS. Next, 50 μl of a cell suspension with a concentration of 5 x $10^5$/ml was added in the sample chamber. The samples were centrifuged at 500 rpm during 10 minutes. The sedimented cells were assessed for IdUrd incorporation by immunoperoxidase staining.

**Cytocentrifugation of agar cultures** The method used for cytocentrifugation of agar cultures is a modification of the method described by Baines.[17] Cultures were cut into halves and shaken into G-PBS. Each half was lifted onto a 76- x 26-mm alcohol-cleaned glass slide and covered with a cellulose acetate strip (Sepraphore; Gelman Sciences, Ann Arbor, MI). Next, a piece of filter paper (Schleicher & Schuell, Dassel, Germany) was put on top, followed by a filter card. The cultures were partially dried during 30 to 60 minutes at room temperature. Subsequently, the slides were put into cytoclips together with the plastic sample chambers. They were centrifuged in a cytospin centrifuge

at 1500 rpm for 10 minutes. After removel of the filters, a thin agar layer, in which the cells were embedded, remained on the glass slide.

#### Detection of incorporated IdUrd by indirect immunoperoxidase staining

The method used to detect incorporated IdUrd was based on a previously published protocol developed for flow cytometry.[13] A line was drawn around the sedimented cells or dried agar cultures with a Dako Pen (Dakopatts, Glostrup, Denmark) to keep the solutions on the slides. After an initial wash step in PBS, a 1 mg/ml pepsine (Serva, Heidelberg, Germany) 2 N HCl solution was applied to make the DNA accessible for the antibodies and to neutralize endogenous peroxidase activity in the cytoplasm. After 30 minutes, 0.1 M $Na_2B_4O_7$ was added for neutralization. Next, pooled human serum (PHS) (20% in PBS) was applied to block aspecific binding sites. Subsequently, a specific monoclonal anti-IdUrd antibody produced in our institute (HN-IU; dilution 1:10) was added.[12] Binding of the anti-IdUrd antibody was visualized by adding the following antibodies: peroxidase-conjugated rabbit anti-mouse immunoglobulins (dilution 1:100; Dakopatts), peroxidase-conjugated goat antirabbit immunoglobulins (dilution 1:250; ICN Biomedicals, Costa Mesa, CA), and peroxidase-conjugated rabbit antigoat immunoglobulins (dilution 1:250; ICN Biomedicals) Slides were washed in PBS in between each step. The incorporated, labeled IdUrd was visualized with 0.5 mg/ml 3,3-diaminobenzidin tetrahydrochloride (DAB) (Sigma) and 0.015% $H_2O_2$ at pH 7.8 in PBS. After 10 to 12 minutes incubation at 37°C, the slides were washed by running them under tap water for 10 minutes, counterstained with hematoxylin solution (Merck, Darmstadt, Germany), and fixated with alcohol (70, 80, 90, 96, 100, 100, and 100%) and xylene substitute (Shandon). The slides were covered with Malinol (Schmid, Köngen, Germany) and a 24- x 50-mm glass slide for microscopical examination In each experiment, a sample not labeled with IdUrd was identically treated and served as negative control.

**Scoring** A cell was considered $IdUrd^+$ when its nucleus contained one or more brown-black spots or when the nucleus was colored totally brown-black Aggregates were considered positive, when >50% of the cells in the aggregate were positive for IdUrd. Per experiment, 100 to 200 aggregates were analyzed. The percentage of positive aggregates was calculated as:

$$\frac{\text{no. of } IdUrd^+ \text{ aggregates}}{\text{no. of } IdUrd^+ \text{ aggregates} + \text{no. of } IdUrd^- \text{ aggregates}} \times 100$$

All experiments were analyzed by one individual.

**Thymidine suicide** A modification of the method of Becker et al. was used for thymidine suicide.[3] Cells ($10^4$/ml) were suspended in IMDM supplemented with 20% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin, and half of the specimen was exposed for 30 minutes to $^3$H-TdR (25 Ci/mM and 1 mCi/ml) at 37°C. The final concentration was 100 µCi/ml. Subsequently, cells were

washed three times with cold G-PBS, containing 100 μg cold thymidine per ml. The remaining cell pellet was resuspended in FCS and used for clonogenic assay. The other half of the cells was not exposed to $^3$H-TdR; the cells were incubated, washed, and plated in agar identically to the suicided specimen and served as controls. The percentage of progenitor cells in S-phase was calculated as.

$$\frac{\text{no. of control aggregates - no. of aggregates after } ^3\text{H-TdR exposure}}{\text{no. of control aggregates}} \times 100$$

**Molt-4** The human (mycoplasma-free) leukemic cell line Molt-4 was cultured in RPMI 1640 (Flow Laboratories) supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamin (Flow Laboratories) in a 5% $CO_2$-humidified atmosphere at 37°C. The cell density was 0.25 to 1.0 x $10^6$ cells/ml. Exponentially growing cells were either exposed to IdUrd (40 μM), $^3$H-TdR (100 μCi/ml), or medium (control) for 30 min at 37°C. After subsequent washing with cold G-PBS (containing 100 μg cold thymidine per ml), part of the cells was used for counterflow centrifugation. Of the remaining noncentrifuged cells, one fraction was used for clonogenic assay and another was fixed in cold 75% ethanol for DNA analysis.[13]

**Molt-4 clonogenic assay** Cells were cultured in RPMI 1640 supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamin, and 0.3% (w/v) bacto-agar. Per experiment eight 35- x 10-mm dishes containing 500 cells in 0.85 ml medium were cultured at 37°C in a fully humidified atmosphere containing 5% $CO_2$. After 4 days, the aggregates (eight to 32 cells) were counted. Agar cultures of IdUrd labeled cells were dried by cytocentrifugation and used for immunoperoxidase staining.

**Counterflow centrifugation of Molt-4 cells** Counterflow centrifugation (CC) was performed with a multichamber rotor (Curamé 3000; Dijkstra Vereenigde, Amsterdam, The Netherlands) to enrich Molt-4 cells in $G_1$ and S-phase.[18] The elutriation profiles proved to be identical for the different chambers.[18] One chamber contained control cells, a second chamber thymidine-labeled, and a third IdUrd-labeled cells. Cells collected at a decreasing rotor speed showed a progressively larger cell volume and represented distinct populations of cells in transition from $G_1$ phase, through S-phase to $G_2M$ phase. On the basis of DNA histograms, collected fractions were combined to obtain four different fractions representing an 'early' and 'late' $G_1$ fraction and an 'early' and 'mid' S-phase fraction Of each fraction, cells were used for DNA analysis and clonogenic assay.

**Bivariate flowcytometric IdUrd/DNA analysis in suspension** Bivariate staining for IdUrd incorporation and DNA content was performed using the simultaneous proteolytic enzyme digestion and acid denaturation technique.[13,19] The anti-IdUrd HN-IU MoAb and a fluorescein isothiocyanate (FITC)-conjugated IgG goat antimouse second-step antibody (GAM-FITC) (American

Qualex International, La Mirada, CA) were used for detection of IdUrd. Propidium iodide (PI) was used for DNA staining. IdUrd staining of the cell suspensions was always performed in duplicate and all incubation steps were executed in the dark. Non-IdUrd-labeled low-density blood cells served as negative controls. Samples were analyzed on a Coulter Epics Elite (Coulter, Hialeah, FL) flow cytometer equipped with a 40 mW Argon ion laser running at 15 mW. A high-pass filter of 610 nm for red fluorescence (PI), a band-pass filter 525/30 for green fluorescence (FITC), and a dichroic mirror of 550 nm were used. A minimum of 20,000 cells were analyzed in duplicate. The fluorescence signals were recorded on a linear scale in list mode. The labeling index was defined as the percentage of IdUrd-labeled cells

**Bioassay for the detection of residual IdUrd after prolonged labeling** Normal bone marrow cells were suspended in IMDM containing 20% FCS and the combination of IL-3, GM-CSF, and G-CSF. The cells were cultured in an incubator for 3 days to attain a high proportion of proliferating cells. After washing with G-PBS, the pelleted stimulated cells were suspended in thawed supernatants and cultured in an incubator for another 24 hours The used supernatants were collected from the cell suspensions, incubated with 1 μM IdUrd for a period of 1 to 5 days. As control, cells were also exposed to freshly prepared 1 μM IdUrd. After incubation, the cells were washed with G-PBS. The cell pellet was resuspended in FCS and used for cytocentrifugation and clonogenic assay. Subsequently, immunoperoxidase staining was performed to determine whether the supernatants contained sufficient IdUrd to label cells in S-phase.

**Statistics** Values were expressed as mean ± 95% confidence interval (CI) of the mean. After IdUrd labeling, the binomial distribution was applied to calculate the 95% CI of the mean percentage of progenitors in S-phase. Significance of IdUrd concentration level was tested with variance analysis (ANOVA with Duncan-Waller test).[20]

## Results

### Development of the detection of incorporated IdUrd

**Immunoperoxidase staining** The first step was the adaptation of the protocol for IdUrd detection by immunofluorescence developed for FCM.[13] In our hands, it was difficult and time-consuming to identify aggregates in agar cultures when fluoresence was used; we preferred an immunoperoxidase staining technique. This allowed evaluation by light microscopy. All aggregates, whether positive for IdUrd or not, could easily be visualized after counterstaining. A sandwich method of three subsequent additions of peroxidase-conjugated immunoglobulins was chosen. Intensification of the signal with

these three peroxidase-conjugated immunoglobulins proved to be superior to the signal obtained with only one or two peroxidase-conjugated immunoglobulins (data not shown).

Adaptation of the semisolid (agar) culture and culture time

Instead of the usual 2.0 ml, a reduced volume of 0.85 ml per dish was plated. Incorporated IdUrd was more easily accessible for antibodies when the thickness of the agar culture was reduced. Subsequent mitoses of the clonogenic cell are expected to dilute the IdUrd signal, which may result in false-negative colonies. The percentage of $IdUrd^+$ bone marrow colonies after 7 culture days (average colony size=64 cells) was the same as determined after 3 days (average cluster size=16 cells, data not shown). This indicated that the immunoperoxidase staining was at least sensitive enough to detect IdUrd in colonies consisting of maximally 64 cells. All cells present in smaller clusters contained at least three spots, whereas most, but not always all, cells of the larger colonies contained one or a few spots (Fig. 1).

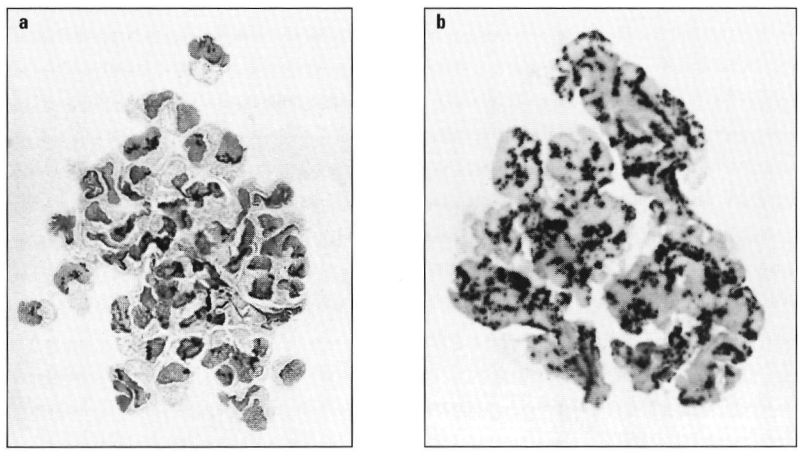


**Figure 1** Examples of $IdUrd^+$ aggregates.
**a** Normal CFU-GM cultured after 24-hour labeling with 1 μM IdUrd.
**b** Molt-4 aggregate cultured after pulse labeling with 40 μM IdUrd.

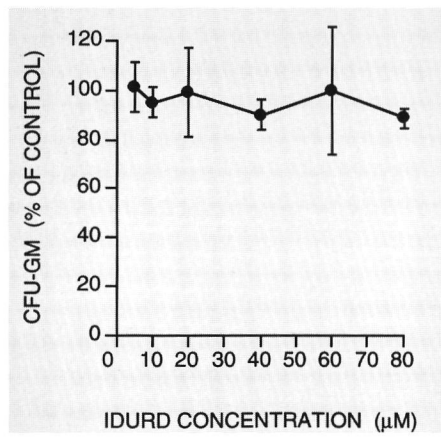


**Figure 2** Toxicity of IdUrd pulse-labeling with IL-3, GM-CSF, and G-CSF stimulated CFU-GM. Normal bone marrow cells were labeled with various concentrations of IdUrd for 2 hours. Subsequently, the number of CFU-GM was determined and expressed as a percentage of the control cultures (incubated without IdUrd). The graphic represent the mean percentage (± 95% CI) of three normal bone marrows.

Toxicity The IdUrd toxicity was tested on three different normal bone marrows. The cells were cultured for 4 days in the presence of the combination of IL-3, GM-CSF, and G-CSF to obtain a high proportion of cycling progenitors. IdUrd was added during the last 2 hours of incubation. The number of CFU-GM was not significantly reduced, even after incubation with 80 μM IdUrd (Fig. 2). However, the size of the aggregates cultured after incubation with 80 μM IdUrd was smaller compared to control cultures. Therefore, 80 μM was considered to be toxic. In addition, peroxidase staining was performed on sedimented cells of the suspensions after labeling with the different IdUrd concentrations. All samples contained $IdUrd^+$ cells. Part of the cells labeled with 80 μM were covered with a brown smear. It appeared as if their nuclei were no longer intact. This, again, suggested toxicity.

Signal intensity Cells were pulse-labeled for a period of 2 hours. Because of an insufficient amount of incorporated IdUrd, a shorter period may underestimate the number of clonogenic cells in S-phase, whereas a longer period may result in overestimation. Pulse-labeling with 5 μM IdUrd resulted in a significantly lower percentage of positive aggregates when compared to the other tested IdUrd concentrations. The mean (±95% CI) positive percentage was 22±20 after pulse labeling with 5 μM vs. 42±8, 37±12, and 45±12 after labeling with 10, 20, and 40 μM respectively (Duncan-Waller; $n = 3, p<0.05$) (Fig. 3). The percentages of positive aggregates after labeling with 10, 20, and 40 μM IdUrd were not significantly different. The lower percentage of positive aggregates found after 80 μM labeling suggested toxicity towards colony-

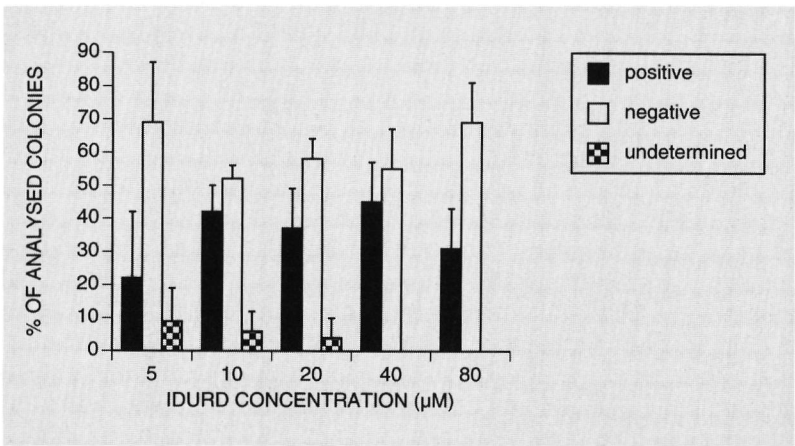


**Figure 3** Signal intensity after IdUrd pulse labeling. Normal bone marrow cells from three donors were labeled with various concentrations of IdUrd for 2 hours. Subsequently, clonogenic assays were performed. Cultures were ended after 6 days and the incorporated IdUrd was detected by immunoperoxidase staining. The diagram shows the mean (± 95% CI) percentage of positive and negative colonies for each IdUrd concentration. Colonies of which the brown staining was too vague to determine whether they were positive or negative are represented as undetermined.

forming cells that incorporated IdUrd After labeling with 5, 10 and 20 μM IdUrd, the brown staining in a small percentage (<10%) of the aggregates was too vague to determine whether they were positive or negative Taking both toxicity and signal intensity into account, 40 μM was considered to be the optimal IdUrd concentration for pulse-labeling

**Sensitivity** Molt-4 cells were used to check whether all clonogenic cells in S-phase were sufficiently pulse-labeled with IdUrd to be detected by immunoperoxidase staining If not, an underestimation of the percentage of colony-forming cells in S-phase may occur In contrast to normal bone marrow cells, Molt-4 cells have a high plating efficiency (20 to 50%) Therefore, cell cycle analysis of the total cell population can be considered to approximate the cycling status of the colony-forming cells The percentage Molt-4 cells in S-phase was determined by FCM using IdUrd incorporation After pulse labeling, 40 9% of the overall Molt-4 cell population was in S-phase, whereas 45 6% (41 1 to 50 3, mean±95% CI) of the colonies were IdUrd$^+$ (Table 1)

**Table 1** Comparison of the percentage of clonogenic Molt-4 cells in S-phase determined by $^3$H-TdR Suicide and IdUrd pulse-labeling in CC fractions containing different percentages of S-phase cells

| CC fraction[a] | % S-phase cells[b] | CFU-Molt[c] Control | CFU-Molt[c] $^3$H-TdR | Suicide index (%)[d] | IdUrd$^+$ CFU-Molt(%)[e] |
|---|---|---|---|---|---|
| Total | 40 9 | 106 6±7 6 | 59 8±9 0 | 43 9<br>28 3 - 59 5 | 45 6<br>41 1 - 50 3 |
| 1 | 13 2 | 14 6±2 0 | 15 3±3 2 | -4 8<br>-40 4 - 30 8 | 14 8<br>11 4 - 19 0 |
| 2 | 27 8 | 31 6±5 6 | 26 0±2 6 | 17 7<br>-8 2 - 43 6 | 30 6<br>25 5 - 36 2 |
| 3 | 45 8 | 65 9±4 2 | 26 0±3 4 | 60 5<br>49 0 - 72 0 | 52 2<br>46 0 - 58 4 |
| 4 | 69 9 | 92 5±8 0 | 11 1±3 2 | 88 0<br>75 9 - 100 1 | 70 1<br>64 9 - 74 8 |

a Fractions (1 to 4) obtained with CC at decreasing rotor spead The total fraction represents Molt-4 cells that were not elutriated

b Percentage of Molt-4 cells in S-phase determined by IdUrd incorporation

c Mean number (± 95% CI, $n$ = 8 cultures) of Molt-4 colonies / 500 cells

d Mean percentage (± 95% CI, $n$ = 8 cultures) of CFU-Molt-4 in S-phase determined by $^3$H-TdR suicide

e Mean percentage (± 95% CI, $n$ = 8 cultures) of CFU-Molt-4 in S-phase determined by IdUrd pulse-labeling

**Table 2** Comparison of the percentage of CFU-GM in S-phase determined by $^3$H-TdR suicide and IdUrd pulse-labeling in four normal bone marrows

| Case no | Culture condition[b] | CFU-GM[a] Control | CFU-GM[a] $^3$H-TdR | Suicide index (%)[c] | IdUrd$^+$ CFU-GM (%)[d] |
|---|---|---|---|---|---|
| 1 | Unstimulated | 154 3 ± 20.9 | 189.5 ± 19 4 | -22 8<br>-48 8 - 3 2 | 4 2<br>1 8 - 9 5 |
| | Stimulated | 409 0 ± 34.3 | 343 7 ± 63 7 | 16 0<br>-7 9 - 39 9 | 33.3<br>27 5 - 39 8 |
| 2 | Unstimulated | 108.7 ± 18.3 | 107 3 ± 5.2 | 1 3<br>-20.3 - 22 9 | 8 3<br>4 4 - 15 0 |
| | Stimulated | 328.7 ± 12.7 | 163 3 ± 19 2 | 50 3<br>40 7 - 59.6 | 56.5<br>49 3 - 63 4 |
| 3 | Unstimulated | 98 5 ± 20 0 | 96 0 ± 2 8 | 2 5<br>-20 6 - 25.6 | 10.4<br>6 6 - 16 0 |
| | Stimulated | 214.0 ± 71 2 | 64 7 ± 15 0 | 69 8<br>29 6 - 110 0 | 48.2<br>42 1 - 54 4 |
| 4 | Fresh | 446 5 ± 84 9 | 293 5 ± 31 0 | 34 2<br>8.2 - 60.2 | 20 3<br>14.2 - 28.3 |
| | Unstimulated | 176 0 ± 15.1 | 164.0 ± 36.7 | 6.8<br>-22.6 - 36 2 | 9.3<br>5 6 - 15 1 |
| | Stimulated | 140.7 ± 7.4 | 63 0 ± 11 0 | 55 2<br>34.4 - 76.0 | 36.4<br>30 4 - 42.9 |

**a** Mean number of CFU-GM / 0 3 x $10^5$ nucleated cells (± 95% CI, $n$ = 6).

**b** Thawed normal bone marrow cells were tested 4 days after liquid culture in the absence (unstimulated) or presence (stimulated) of the combination of IL-3, GM-CSF, and G-CSF In case 4, freshly obtained bone marrow cells were also tested

**c** Percentage (± 95% CI, $n$ = 6 cultures) of CFU-GM in S-phase determined by $^3$H-TdR suicide.

**d** Percentage (± 95% CI, $n$ = 200 aggregates) of CFU-GM in S-phase determined by IdUrd pulse-labeling.

In addition, Molt-4 cells were elutriated to create cell populations with an increasing number of S-phase cells. This allowed evaluation of the accuracy of IdUrd pulse-labeling under different conditions. The percentage of S-phase cells increased from 13.2% in fraction 1 to 69.9% in fraction 4 (Table 1). The percentage of IdUrd$^+$ CFU-Molt-4 increased from 14.8% (11.4 - 29.0) in fraction 1 to 70.1% (64.9- 74.8) in fraction 4. The percentage of IdUrd$^+$ colonies corresponded strongly with the percentage S-phase cells.

**Comparison of $^3$H-TdR suicide and IdUrd pulse-labeling** IdUrd pulse-labeling was compared with $^3$H-TdR suicide, which is considered to be the most reliable suicide technique [4] Unfractionated Molt-4 cells and Molt-4 cell fractions containing different quantities of S-phase cells were tested The fractions were obtained by counterflow centrifugation. Comparison between the suicide index and the percentage of IdUrd$^+$ CFU-Molt-4 revealed no significant differences for the five tested samples (Table 1). The wide confidence intervals of the suicide technique contrasted with the small confidence intervals obtained with IdUrd pulse-labeling.

In a second set of experiments, normal bone marrow cells were cultured for 4 days in the absence or presence of the combination of IL-3, GM-CSF, and G-CSF to generate colony-forming cells with a low and high proliferative activity, respectively. In unstimulated thawed bone marrow the percentage of CFU-GM in S-phase varied from -23 to 7% when determined with thymidine suicide and from 4 to 10% when assessed with IdUrd pulse-labeling (Table 2). After stimulation with the combination of growth factors, the percentage of CFU-GM in S-phase ranged from 16 to 70% ($^3$H-TdR suicide) and 33 to 56% (IdUrd pulse-labeling). No significant differences were observed between the percentages obtained with $^3$H-TdR suicide and IdUrd labeling. The confidence intervals of the suicide index were again much wider than those of the IdUrd pulse-labeling. The fresh bone marrow sample showed a higher percentage of clonogenic cells in S-phase with both methods, probably owing to the presence of viable CSF-producing monocytes in this sample (Table 2, sample 4).

## Prolonged labeling

**Toxicity of various concentrations of IdUrd during 5-day incubation** The toxicity was tested on three normal bone marrows. Cells were stimulated with the combination of IL-3, GM-CSF, and G-CSF to obtain a high proportion of proliferating cells. It appeared that prolonged labeling with IdUrd concentrations of 2 μM and higher was toxic for CFU-GM (Fig 4). The curves obtained with 2, 3, 4, and 5 μM showed no further decrease in CFU-GM number after 4- and 5-day exposure. This could indicate that IdUrd concentrations had decreased below toxic levels. Therefore, the concentration of IdUrd in time was investigated

**Sensitivity** Theoretically, 1 μM IdUrd could be too low to label all clonogenic cells in S-phase. Therefore, the percentage of positive colonies obtained after 24-hour labeling with 5 μM (the highest nontoxic concentration) as well as 1 μM and 0.5 μM were compared. It appeared that about 75% of the colonies were IdUrd$^+$ at all three concentrations tested (Fig. 5). This demonstrated that 1 μM IdUrd was sufficient to label all cycling cells during a period of at least 24 hours.

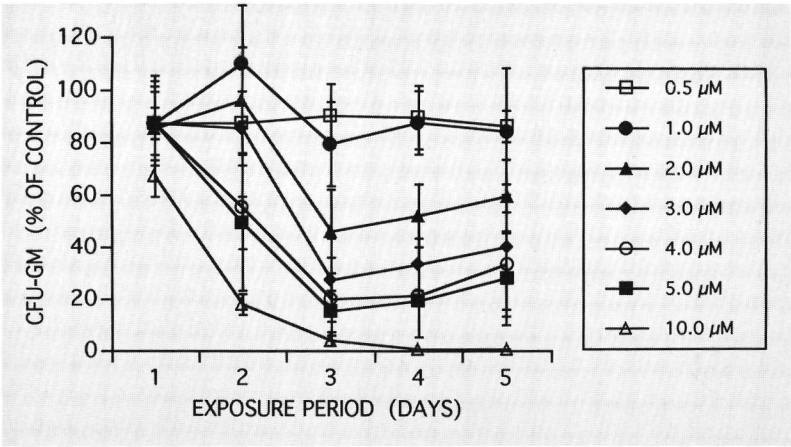


**Figure 4** Toxicity of prolonged IdUrd labeling toward CFU-GM. Normal bone marrow cells were labeled with various concentrations of IdUrd for up to 5 days. Subsequently, the number of CFU-GM was determined and expressed as a percentage of the control cultures (100%) (incubated without IdUrd). The graphic represent the mean percentage (±95% CI) of three normal bone marrows.

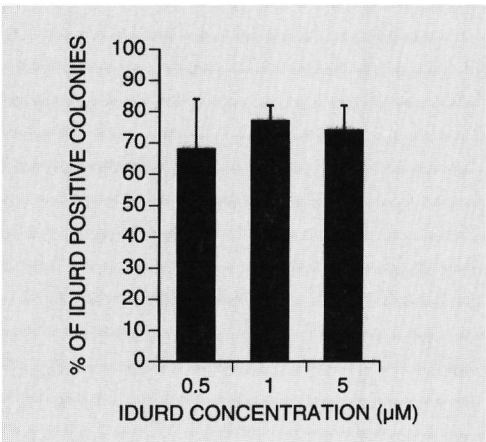


**Figure 5** Signal intensity after prolonged IdUrd labeling. Normal bone marrow cells from three donors were labeled with various concentrations of IdUrd for 24 hours. Subsequently, clonogenic assays were performed. Cultures were ended after 6 days and the incorporated IdUrd was detected by immunoperoxidase staining. The diagram shows the mean (±95% CI) percentage of IdUrd$^+$ colonies for each concentration of IdUrd.

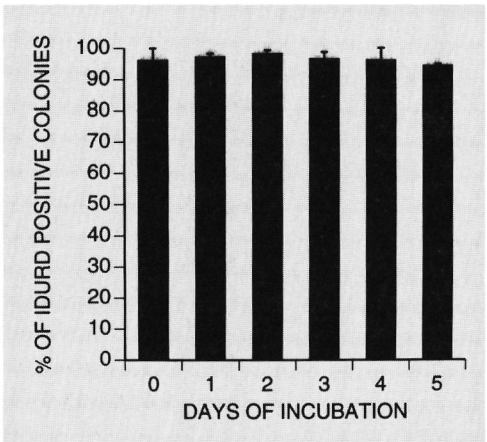


**Figure 6** IdUrd concentration in time. IL-3, GM-CSF, and G-CSF-stimulated normal bone marrow cells were incubated with supernatants collected after incubation of cell suspensions with 1 μM IdUrd for a period of 1 to 5 days. As control, cells were also incubated with a freshly prepared 1 μM IdUrd suspension (=day 0). After 24 hours, clonogenic assays were performed. Cultures were ended after 6 days and IdUrd was detected by immunoperoxidase staining. The mean (±95% CI) percentage of IdUrd$^+$ colonies reflects the residual amount of IdUrd which could be incorporated.

**IdUrd concentration in time** The toxicity study indicated that 1 μM IdUrd was the highest concentration that could be safely used for prolonged labeling IdUrd can be inactivated, especially in media containing FCS not inactivated by heat (data not shown). The media used in this study contained heat-inactivated FCS. Since 1 μM or less IdUrd was below the detection limit of high-performance liquid chromatography (HPLC), a bioassay was performed. It appeared that IdUrd sufficient to label colony-forming cells for up to 5 days was present in the supernatants. The percentages of positive colonies were the same as obtained after exposure to a freshly prepared 1 μM solution (Fig. 6). However, the intensity of the signal in the colonies, cultured after incubation with the supernatant from day 4 and day 5, was reduced

## Discussion

Suicide techniques are available to study the kinetics of colony-forming cells *in vitro*. The fractional reduction in cloning efficiency after exposure to $^{3}$H-TdR, HU or Ara-C may not always estimate the percentage of S-phase cells correctly.[2,4,5] Calculation of the suicide index is based on the subtraction and division of colony numbers. This is a major disadvantage of all suicide techniques. The confidence intervals of colony-forming assays are substantial. A 95% CI of ~20% is not uncommon.[3,21] Statistically, this has a great impact on the accuracy of the suicide index Calculation of the 95% CI of the suicide index of some publications revealed a variation up to 30%.[3,21]

Nonradioactive IdUrd appears to be an attractive alternative for determining the proportion of colony-forming cells in S-phase. In contrast to the suicide techniques, the percentage of IdUrd$^{+}$ colonies per culture dish is independent of the variation in colony numbers. In stead, the exactness of the estimated percentage S-phase cells depends on the total number of analyzed colonies.[20]

After adaptation of a protocol developed for immunological detection of IdUrd by FCM, IdUrd$^{+}$ colonies could easily be detected in agar cultures (Fig.1). The immunoperoxidase staining technique was sensitive enough to detect IdUrd in colonies consisting of maximally 64 cells.

Toxicity and sensitivity experiments indicated that a 2-hour exposure to 40 μM IdUrd was optimal for pulse-labeling Underestimation of the percentage S-phase cells due to insufficient incorporated IdUrd was excluded by extensive studies with Molt-4 cells (Table 1). Molt-4 cells and normal bone marrow cells with varying percentages of cells in S-phase were used to compare IdUrd pulse-labeling with $^{3}$H-TdR suicide. The mean percentage of S-phase cells determined with IdUrd labeling was not significantly different from the percentage obtained with $^{3}$H-TdR suicide (Tables 1 and 2). Due to the inevitable variation in colony numbers, the extent of the 95% CI of the suicide index was substantial. Based on the evaluation of only 200 aggregates, a much smaller confidence interval was calculated in case of IdUrd pulse-labeling. Thus, IdUrd pulse-labeling appeared to be more accurate than $^{3}$H-TdR suicide. A negative suicide index, which means higher colony numbers in the

$^{3}$H-TdR exposed fraction, was observed in two samples. Both contained a low percentage of S-phase cells (4.2% and 14.8%, respectively when determined with IdUrd pulse-labeling) The relatively small number of $^{3}$H-TdR killed cells fell probably within the variation of the colony-forming assays. Other authors made identical observations.[3,21] It again shows a limitation of suicide techniques.

Toxicity and inactivation of IdUrd and sensitivity of the IdUrd detection were evaluated to determine the optimal concentration for prolonged labeling. It appeared feasible to determine the proportion of cycling clonogenic cells for a period of at least 5 days during which a concentration of 1 μM IdUrd was used to label the cells Thus, prolonged IdUrd labeling provides the unique opportunity to determine the proliferating fraction of progenitor cells. With the traditional techniques, only the fraction of S-phase cells can be determined.

During DNA repair, both $^{3}$H-TdR and IdUrd will be incorporated into non-S-phase cells. In theory, this could result in an overestimation of cells in S-phase. However, the number of repaired sites is relatively small compared to the doubling of DNA that occurs during S-phase. This suggests only a minor or even no detectable effect of DNA repair on the estimation of cells in S-phase. In some leukemic samples, we observed that even during 3-day labeling with 1 μM IdUrd, <5% of the leukemic clonogenic cells were IdUrd$^{+}$ (data not shown). This additionally supports the limited influence of DNA repair on the estimation of S-phase cells

Prolonged and pulse-labeling with IdUrd can be applied to study the proliferative behavior of progenitors under physiological and pathological conditions. For example, the effects of growth factors on the *in vitro* proliferation of normal and leukemic colony-forming cells can be analyzed. Furthermore, cycling data can be correlated to the cytotoxicity of chemotherapeutic drugs. IdUrd can safely be administered to humans.[11,12] Kinetic analysis of *in vivo* labeled progenitors seems feasible, and the first clinical data are currently collected in our institute.

The present study shows that the percentage of colony-forming cells in S-phase can be determined more accurately with IdUrd pulse-labeling than with suicide techniques. In addition, prolonged IdUrd labeling makes it possible to study the proliferating fraction of progenitor cells for at least 5 days.

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# Chapter 5

***Hematopoietic growth factors increase the fraction of cycling leukemic clonogenic cells and reduce the kinetic resistance to cytosine arabinoside in vitro***

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Submitted

## Summary

A recently developed *in vitro* 3-day iododeoxyuridine (IdUrd) labeling technique was used to investigate whether growth factors may influence the kinetics of leukemic clonogenic cells (CFU-L) and thus may alter the sensitivity to the cell cycle-specific drug cytosine arabinoside (Ara-C). In addition, results were compared with the effects on normal progenitors (CFU-GM). Liquid cultures were performed in the absence and presence of preselected combinations of interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). The median percentage of cycling CFU-L increased significantly ($p = 0.009$) from 8.5% without growth factors to 87.5% with growth factors in five non-spontaneously growing leukemias. The Ara-C sensitivity increased median 63-fold (range 14-217). The relative percentage of surviving $IdUrd^+$ progenitors decreased with increasing concentrations of Ara-C. This indicated that Ara-C was preferentially cytotoxic to CFU-L going through S-phase. Two spontaneously growing leukemias demonstrated a high proportion of CFU-L in S-phase (93% and 99.3%) and were highly Ara-C sensitive regardless whether exposed to the combination of IL-3, GM-CSF, and G-CSF or not. Leukemias with a comparable proportion of cycling CFU-L were not equally Ara-C sensitive, suggesting that other than cell cycle-related toxicity was operational as well. The three tested normal bone marrows showed a more uniform response pattern. Even in the absence of growth factors, 70 to 80% of normal CFU-GM were cycling. The percentage of cycling CFU-GM increased to 100% in the presence of the combination of IL-3, GM-CSF, and G-CSF and resulted in median 4 3-fold (range 1 4-5.3) enhancement of Ara-C toxicity.

Our data indicate that the increment of proliferating leukemic clonogenic cells by growth factors may contribute to overcome kinetic resistance to Ara-C in AML patients, especially in the setting of protocols with continuous exposure to Ara-C.

## Introduction

A small subset of clonogenic cells within the leukemic population is held responsible for the maintenance of the leukemic clone.[1-4] Antileukemic therapy should focus on the eradication of these leukemic progenitor cells (CFU-L) with relative sparing of normal bone marrow stem cells.

The antileukemic drug cytosine arabinoside (Ara-C) interferes with DNA synthesis and is cytotoxic to S-phase cells.[5] However, a substantial number of leukemic blasts is non- or very slowly cycling and therefore may be resistant to Ara-C.[6-10] Apart from metabolic resistance, this kinetic resistance is considered to be an important mechanism for Ara-C resistance in acute myeloid leukemia (AML).[11] Theoretically, clinical results may improve by circumvention of kinetic resistance through proliferation induction. As effective stimulators of leukemic blasts, interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) are candidates for proliferation induction in AML.[12-17]

We and others demonstrated that *in vitro* IL-3, GM-CSF, and G-CSF may enhance Ara-C toxicity towards CFU-L.[17-25] In specific AML cases, the most optimal promotion of clonogenic cell growth and improvement of Ara-C toxicity was achieved by exposure to individually selected growth factors.[17] These observations suggest that growth factors may increase the number of CFU-L in S-phase However, little is known about the effect of growth factors on the kinetics of leukemic *progenitor* cells. One *in vitro* study determined the influence of GM-CSF on the percentage of clonogenic cells in S-phase in three leukemic samples.[19] In these cases, GM-CSF increased the S-phase fraction of CFU-L from 39.3 to 67% as demonstrated by Ara-C suicide technique. By thymidine suicide technique, a significant increase of clonogenic leukemic cells in S phase from 33 to 55% was observed in one patient studied before and after 18 h of GM-CSF therapy.[26]

Previously, we developed an *in vitro* technique to measure the fraction of actively cycling colony-forming cells by prolonged iododeoxyuridine (IdUrd) labeling.[27] The nonradioactive thymidine analog IdUrd is incorporated into DNA during S-phase and detected by an anti-IdUrd antibody. When a colony-forming assay is performed following the IdUrd exposure, incorporated IdUrd can be visualized by immunoperoxidase staining. Detection of IdUrd in the cells of individual colonies indicates that these colonies originated from colony-forming cells which passed the S-phase during the labeling period.

In the present study, this new IdUrd technique was used to investigate the effects of IL-3, GM-CSF, and G-CSF on the kinetics of CFU-L. Since kinetic studies indicate that the median total cell cycle duration is about 70 hours for AML blasts,[10] a 3-day continuous IdUrd labeling period was chosen. Based upon our previous results,[17] specific AML cases were tested in the absence and presence of individually adapted growth factors shown to result in optimal clonogenic cell growth. For comparison, the influence of IL-3, GM-CSF, and

G-CSF on normal CFU-GM was also evaluated. Finally, the Ara-C sensitivity of CFU-L and CFU-GM was determined and related to the kinetic responses.

Our data indicate that individually selected colony-stimulating factors increase the number of cycling CFU-L and reduce the kinetic resistance to Ara-C.

## Materials and Methods

**Patients** Bone marrow samples from seven previously untreated AML patients were tested. The diagnosis according to the French-American-British (FAB) classification was M2 (n = 4) and M4 (n = 3) (Table 1).[28] The bone marrow samples contained median 61% blasts (range 35-94). Normal bone marrow was obtained from three healthy bone marrow donors. Informed consent was given in all cases.

**Bone marrow preparation, cryopreservation, thawing** Normal and leukemic bone marrow cells were collected in sterile buffered acid-citrate dextrose (pH = 7.0) The normal and leukemic samples were enriched for clonogenic cells by flotation centrifugation or centrifugation on a Ficoll (1.085 g/ml) density gradient (Sigma, St Louis, Missouri) respectively.[29] Normal bone marrow cells with a density of <1.067 g/ml and leukemic interphase cells were cryopreserved in liquid nitrogen using a temperature-controlled freezer (Kryo 10; Planer Biomed, Sunbury, Middlesex, UK). Vials contained 5-20 x $10^6$ cells suspended in Iscove's medium (Flow Laboratories, Irvine, Scotland), supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone, Logan, Utah), 50 IU/ml penicillin, 50 µg/ml streptomycin (both Flow Laboratories) and 10% dimethylsulfoxide (DMSO). Just prior to the experiments, cells were thawed in a 37°C waterbath, resulting in a recovery of >90%. Freezing and thawing procedures have been described in detail elsewhere.[30]

**Human recombinant growth factors** Human recombinant IL-3 and GM-CSF were kindly donated by Sandoz BV (Uden, The Netherlands). Human recombinant G-CSF was a generous gift from Behring (Marburg, Germany). The final concentrations for plateau stimulation in both liquid and semisolid cultures were 40 ng/ml, 20 ng/ml, and 5 ng/ml respectively

**Liquid cultures with Ara-C and IdUrd** Thawed leukemic and normal cells ($10^4$/ml) were cultured in liquid in the absence and presence of preselected growth factors for a period of 5 days. The suspensions consisted of Iscove's medium supplemented with 20% FCS, 50 IU/ml penicillin, and 50 µg/ml streptomycin. Previous experiments showed a median survival of the clonogenic cells of more than 100% under these conditions.[18] The choice of the added factor(s) was based on previous experiments in which the responsiveness to IL-3, GM-CSF, and G-CSF, both alone and in combination, was established in liquid cultures.[17] Growth factor(s) resulting in the highest number of CFU-L were selected for the leukemic samples. The combination of IL-3,

GM-CSF, and G-CSF yielded the best results in normal bone marrow samples Cells were allowed to recover from thawing and to adjust to the culture conditions for 2 days. During the next 3 days of incubation, 1 μM of IdUrd (Sigma) was added to duplicate cultures. These samples were used to determine the number of cycling clonogenic cells and served as the 100% control growth. Previous experiments showed that 1 μM of IdUrd was not toxic for myeloid progenitor cells.[27] To parallel liquid cultures, various Ara-C concentrations (Upjohn, Kalamazoo, Michigan) were added during the last 3 incubation days. The final concentrations varied from $10^{-10}$ to $10^{-5}$ M Ara-C. Dilutions were prepared from freshly thawed Ara-C stock solutions. The drug was checked for concentration, purity, and stability by high-performance liquid chromatography.[31] Four leukemic samples were also exposed to Ara-C in the presence of 1 μM IdUrd to determine the percentage of cycling CFU-L at certain Ara-C concentrations. After culture in an incubator (37°C, 5% $CO_2$ in air and fully humidified atmosphere), the cells were washed with glucose-phosphate-buffered saline (G-PBS) and used for clonogenic assay.

**Clonogenic assay (CFU-GM/L)** Cells were cultured in Iscove's medium supplemented with 20% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 0 3% (w/v) bacto-agar (Difco, Detroit, Michigan). Cells were stimulated with the combination of IL-3, GM-CSF, and G-CSF. Duplicates were cultured in 35- x 10-mm culture dishes (Costar, Cambridge, Massachusetts) at 37°C in a fully humidified atmosphere containing 5% $CO_2$ in air. Per dish, a total volume of 0.85 ml was plated. The cultures were evaluated after 5 to 7 days, when aggregates consisted of 20-60 cells. This enabled a reliable detection of IdUrd in the aggregates.[27] The CFU-L aggregates consisted of immature cells. The CFU-GM aggregates usually contained mature granulocytes and/or macrophages. After enumerating the clusters (5-40 cells) and colonies (>40 cells), the agar cultures were dried by cytocentrifugation and used for immunoperoxidase staining.

**Cytocentrifugation of agar cultures** The method used is a modification of the method described by Baines.[32] Cultures were cut into halves and shaken into G-PBS. Each half was lifted onto a 76- x 26-mm glass slide and covered with a cellulose acetate strip (Sepraphore, Gelman Sciences, Ann Arbor, Michigan). Next, a piece of filter paper (Schleicher & Schuell, Dassel, Germany) was put on top, followed by a filter card. The slides were centrifuged in a cytospin centrifuge at 1500 rpm for 10 minutes (Shandon, Pittsburgh, Pennsylvania). After removal of the filters, a thin agar layer, in which the cells were embedded, remained on the glass slide.

**Detection of incorporated IdUrd by indirect immunoperoxidase staining**

The method used to detect incorporated IdUrd was based on a previously published protocol developed for flow cytometry and has been described in detail elsewhere.[27,33] A 1 mg/ml pepsine (Serva, Heidelberg, Germany) 2 N HCl solution was applied on the dried agar cultures. After 30 minutes, 0.1 M $Na_2B_4O_7$ was added for neutralization. Next, pooled human serum (PHS)

(20% in PBS) was applied to block aspecific binding sites. Subsequently, a specific monoclonal anti-IdUrd antibody produced in our institute (HN-IU, dilution 1:10) was added.[34] Binding of the anti-IdUrd antibody was detected by subsequent addition of peroxidase-conjugated rabbit antimouse immunoglobulins (dilution 1:100, Dakopatts, Glostrup, Denmark), peroxidase-conjugated goat antirabbit immunoglobulins (dilution 1:250, ICN Biomedicals, Costa Mesa, California), and peroxidase-conjugated rabbit anti-goat immunoglobulins (dilution 1 250, ICN Biomedicals). The incorporated, labeled IdUrd was visualized with 0.5 mg/ml 3,3-diaminobenzidin tetrahydrochloride (DAB) (Sigma), 0.015% $H_2O_2$ at pH 7.8 in PBS. The slides were counterstained with hematoxylin solution (Merck, Darmstadt, Germany), and fixated with alcohol and xylene substitute (Shandon). In each experiment, an $IdUrd^+$ and an $IdUrd^-$ sample were identically treated and served as controls.

**IdUrd scoring** Aggregates were considered $IdUrd^+$ when >50% of the cells in the aggregate were positive for IdUrd. A cell was considered $IdUrd^+$ when its nucleus contained one or more brown-black spots or when the nucleus was totally brown-black colored. Per experiment, 100 to 250 aggregates were analyzed. The percentage of positive aggregates was calculated as:

$$\frac{\text{no. of } IdUrd^+ \text{ aggregates}}{\text{no. of } IdUrd^+ \text{ aggregates} + \text{no. of } IdUrd^- \text{ aggregates}} \times 100$$

All experiments were analyzed by one single individual.

**Curve fitting and statistics** To fit the Ara-C dose response curves, the following formula was used:

$$f(x)^2 = \frac{a}{1+(bx)^c}$$

In this formula $a$ stands for the maximal plating efficiency (100%, measured when the cells are incubated without Ara-C) and $b$ for the Ara-C concentration at which inhibition is 70%; $c$ represents the slope of the curve. The data were fitted according to the Gauss-Newton regression procedure with the use of the least squares criteria.[35] The Ara-C concentrations resulting in 50% inhibition of clonogenicity ($ID_{50}$) were calculated from these curves.
Values were expressed as mean ± 95% confidence interval (CI), as mean ± standard error of the mean (SEM) or as median with range. The binomial distribution was applied to calculate the 95% CI of the mean percentage of $IdUrd^+$ progenitor cells.[36] Group data were compared by Mann-Whitney test.

**Table 1** Relation between factor responsiveness, percentage of cycling clonogenic cells and Ara-C toxicity

| Patient no | FAB type[a] | Spontaneously growing[b] | Added factor[c] | Control growth[d] | IdUrd+[e] | Ara-C $ID_{50}$ x $10^{-8}$ M[f] | Ara-C Sensitivity Increase |
|---|---|---|---|---|---|---|---|
| 1 | M2 | - | No factor | 7900 | 7 3<br>4 5 - 11 7 | 282 | |
| | | | GM-CSF | 21200 | 65 5<br>58 7 - 71 7 | 19 1 | 14 8 x |
| 2 | M2 | - | No factor | 2800 | 2 5<br>1 1 - 5 7 | >1000 | |
| | | | Il-3+ GM-CSF+ G-CSF | 6600 | 89 2<br>83 3 - 91 4 | 4 6 | >217 4 x |
| 3 | M2 | - | No factor | 110 | 37 1<br>28 5 - 46 7 | 97 5 | |
| | | | GM-CSF | 1800 | 87 5<br>82 2 - 91 4 | 3 9 | 25 0 x |
| 4 | M2 | + | No factor | 6100 | 93 0<br>88 0 96 1 | 1 5 | |
| | | | Il-3+ GM-CSF+ G-CSF | 4600 | 97 4<br>93 6 - 99 0 | 2 6 | 0 6 x |
| 5 | M4 | - | No factor | 5000 | 8 5<br>5 2 - 13 8 | 70 4 | |
| | | | GM-CSF+ G-CSF | 23000 | 68 0<br>58 3 - 76 3 | 0 6 | 117 3 x |
| 6 | M4 | - | No factor | 4300 | 22 9<br>17 3 29 6 | 37 8 | |
| | | | GM-CSF | 16200 | 88 5<br>83 3 - 92 2 | 0 6 | 63 0 x |
| 7 | M4 | + | No factor | 4700 | 95 3<br>90 9 97 6 | 0 1 | |
| | | | Il-3+ GM-CSF+ G-CSF | 2800 | 99 3<br>95 9 - 99 9 | 0 1 | 1 0 x |
| 8 | Normal BM | - | No factor | 1000 | 71 3<br>65 1 - 76 8 | 14 9 | |
| | | | Il-3+ GM-CSF+ G-CSF | 2700 | 100 0<br>96 7 - 100 0 | 2 8 | 5 3 x |
| 9 | Normal BM | - | No factor | 1900 | 73 1<br>64 1 - 80 6 | 2 3 | |
| | | | Il-3+ GM-CSF+ G-CSF | 4600 | 100 0<br>97 3 - 100 0 | 1 7 | 1 4 x |
| 10 | Normal BM | - | No factor | 1100 | 81 7<br>75 4 - 86 6 | 5 2 | |
| | | | Il-3+ GM-CSF+ G-CSF | 3600 | 100 0<br>97 9 - 100 0 | 1 2 | 4 3 x |

*a* French-American-British classification [28]
*b* Defined as the formation of aggregates in semisolid culture in the absence of exogenously added growth factors
*c* Growth factor(s) added to the liquid culture
*d* CFU-L/CFU-GM number per 2 x $10^5$ 'input' cells, assessed in duplicate after 5-day liquid culture in the absence of Ara-C
*e* Percentage (± 95% CI) of cycling CFU-L/CFU-GM determined by 3-day IdUrd labeling
*f* Ara-C concentration that inhibited 50% of the CFU-L/CFU-GM growth The values were calculated from survival curves constructed after 3-day exposure to various concentrations of Ara-C

## Results

### Non-spontaneously growing leukemias

Of the seven tested leukemias, five did not form aggregates when cultured in semisolid medium in the absence of exogenously added growth factors (patients 1, 2, 3, 5, and 6, Table 1). These leukemias were considered to be non-spontaneously growing. After incubation with IdUrd for 3 days in the absence of growth factors, a median number of 8.5% (range 2.5 to 37.1) colony-forming cells were $IdUrd^+$ (Table 1). Exposure to Ara-C for 3 days resulted in $ID_{50}$ values ranging from $>10^{-5}$ to $3.8 \times 10^{-7}$ M Ara-C. In the presence of individually selected stimulating factors, the fraction of $IdUrd^+$ aggregates increased significantly ($p = 0.009$) from median 8.5 to 87.5% (range 65.5 to 89.2, Table 1). The $ID_{50}$ values decreased significantly ($p = 0.009$) and ranged from $1.9 \times 10^{-7}$ to $6 \times 10^{-9}$ M Ara-C. Compared to the non-stimulated samples, Ara-C toxicity increased median 63-fold (range 14.8 to 217) in the presence of growth factors.

### Spontaneously growing leukemias

Two of the seven tested leukemias formed aggregates when cultured in semi-solid medium in the absence of exogenously added growth factors (patients 4 and 7, Table 1). Both in the absence and presence of growth factors, a high proportion (93% and 99.3%) of the colony-forming cells of these spontaneously growing leukemias were cycling. Either with or without colony-stimulating factors, the $ID_{50}$ values varied from $2.6 \times 10^{-8}$ to $1 \times 10^{-9}$ M Ara-C after 3-day exposure. No additional increase in Ara-C sensitivity was observed after incubation with growth factors.

### Normal bone marrow

The normal bone marrow samples did not form aggregates in semisolid medium when cultured in the absence of exogenously added growth factors. Three-day IdUrd labeling during liquid culture in the absence of added growth factors resulted in median 73.1% (range 71.3 to 81.7) positive colony-forming cells (Table 1). The $ID_{50}$ values calculated after 3-day Ara-C exposure varied from 2.3 to $14.9 \times 10^{-8}$ M Ara-C. In the presence of growth factors, 100% of the CFU-GM were $IdUrd^+$ ($p = 0.05$, when compared to incubation without growth factors). The corresponding $ID_{50}$ values ranged from 1.2 to $2.8 \times 10^{-8}$ M Ara-C and were not significantly different from the values obtained without stimulation (Table 1, $p = 0.12$).

## Undisturbed Ara-C toxicity in the presence of IdUrd

Ara-C and IdUrd are both incorporated into DNA during S-phase and may interfere with each other. Blasts of two patients were incubated with Ara-C and the combination of Ara-C and 1 μM IdUrd to test the influence of IdUrd on the Ara-C cytotoxicity. Experiments were conducted in the absence and presence of stimulating growth factors. The toxicity curves showed that 1 μM of IdUrd did not influence the cytotoxicity of Ara-C, since curves with and without IdUrd were overlapping in both experiments (Fig. 1).

## Preferential Ara-C toxicity towards S-phase CFU-L

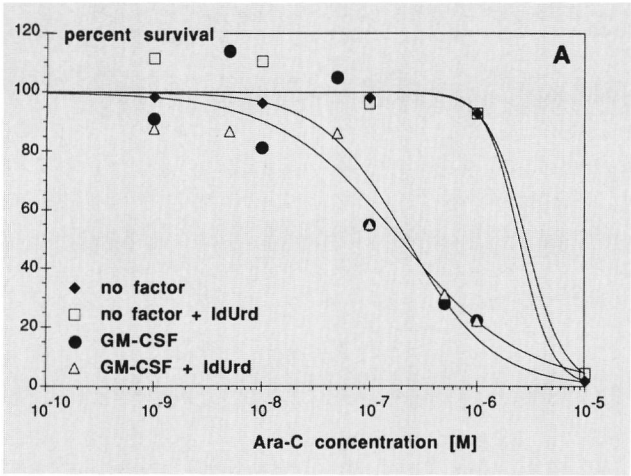
In four patients the S-phase dependent kill of Ara-C was further evaluated. Blasts were incubated with various concentrations of Ara-C and 1 μM IdUrd. Cytospin preparations were made to determine the number of $IdUrd^+$ progenitors after exposure to certain Ara-C concentrations. Samples were tested in the presence of preselected growth factors. Increasing concentrations of Ara-C resulted in a higher kill of CFU-L. The relative percentage of $IdUrd^+$ progenitors decreased with increasing concentrations of Ara-C, indicating that $IdUrd^+$ CFU-L were preferentially killed. A representative example (patient 1) is depicted in Figure 2. These experiments allowed construction of Ara-C survival curves of both the $IdUrd^+$ and $IdUrd^-$ subpopulations. Subsequently, $ID_{50}$ values could be calculated. In all cases, the $IdUrd^+$ CFU-L populations were more sensitive to Ara-C than the $IdUrd^-$ CFU-L populations (Table 2).

**Table 2** Ara-C sensitivity for $IdUrd^+$ and $IdUrd^-$ clonogenic leukemic cells

| Patient no | Added factor[b] | Ara-C $ID_{50} \times 10^{-8}$ M[a] | | |
|---|---|---|---|---|
| | | Overall | $IdUrd^+$ | $IdUrd^-$ |
| 1 | GM-CSF | 19 1 | 7 2 | 140 |
| 2 | IL-3+ GM-CSF+ G-CSF | 4.6 | 3.5 | >10 |
| 5 | GM-CSF+ G-CSF | 0.6 | 0.3 | 0.9 |
| 6 | GM-CSF | 0.6 | 0 4 | >10 |

a Ara-C concentration that inhibited 50% of the CFU-L growth. The values were calculated from survival curves constructed after 3-day exposure to various concentrations of Ara-C

b Growth factor(s) added to the liquid culture.



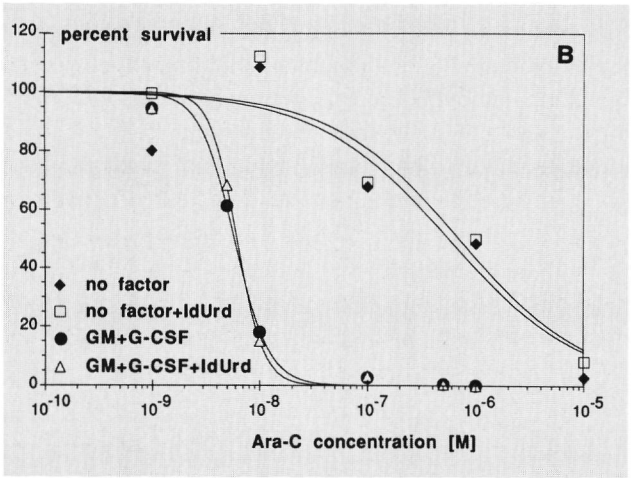




**Figure 1** Ara-C dose survival curves of patient 1 (A) and 5 (B) demonstrating the undisturbed Ara-C toxicity in the presence of IdUrd. In the absence and presence of the indicated growth factors, blasts were exposed to various concentrations of Ara-C in the absence and presence of 1 μM IdUrd. After 3-day Ara-C exposure, the number of surviving CFU-L was determined by clonogenic assay stimulated with the combination of IL-3, GM-CSF, and G-CSF. The CFU-L survival was expressed as a percentage of control cultures.

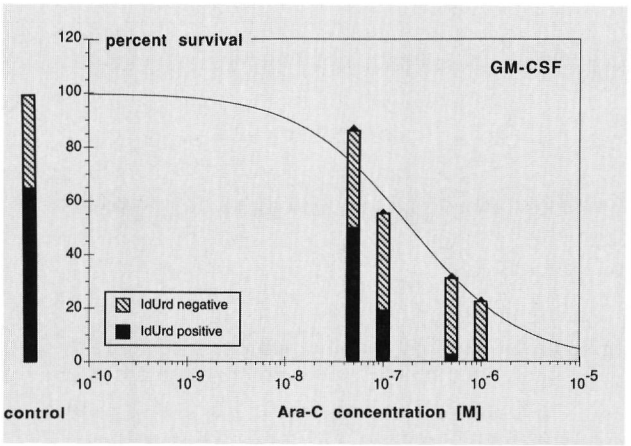



**Figure 2** Ara-C dose survival curve of patient 1 constructed after 3-day exposure to various concentrations of Ara-C in the presence of both GM-CSF and IdUrd. The number of surviving CFU-L was determined by clonogenic assay stimulated with the combination of IL-3, GM-CSF, and G-CSF and expressed as a percentage of control cultures. Each bar represents the relative distribution of IdUrd+ and IdUrd- aggregates at a specific Ara-C concentration. The control was not exposed to Ara-C, but otherwise equally treated.

## Discussion

Augmentation of the cytotoxicity of cycle-specific drugs through recruitment of quiescent leukemic clonogenic cells by growth factors has been studied *in vitro*.[17-25,37,38] Most studies have been performed with Ara-C in combination with GM-CSF and IL-3, although occasionally reports with G-CSF have been published. In almost all studies, the kinetic response to growth factors is assessed in the total *blast* cell population. The percentage of blasts in S-phase is determined by propidium iodide or by the incorporation of $^{3}$H-thymidine ($^{3}$H-TdR) or bromodeoxyuridine (BrdU) into blasts. In contrast, Ara-C toxicity is mainly tested on leukemic *clonogenic* cells. Since the total population of blasts usually comprises <1% clonogenic cells, cell cycle analysis of the entire leukemic cell population may not necessarily reflect the kinetics of the leukemic progenitor cells.[9,19,37] This may explain why the relation between stimulation and increase of Ara-C toxicity was not always obvious. In some cases, enhanced Ara-C toxicity towards CFU-L was observed without a substantially increase in S-phase cells.[20] On the other hand, increased percentages of S-phase cells did not always result in a higher kill by Ara-C.[25,37]

To our knowledge, the present study is the first in which the cycling behaviour of leukemic cells are studied at a clonogenic cell level during a 3-day period. The prolonged IdUrd labeling informs about the CFU-L kinetics during the entire Ara-C exposure period. In non-spontaneously growing leukemias, significantly higher percentages of CFU-L passed S-phase in cultures supplemented with appropriate growth factors compared to CFU-L cultured in the absence of growth factors. This supports the theory that hematopoietic factors play a major role in the regulation of CFU-L proliferation. Proliferation induction with growth factors always corresponded with an increased Ara-C sensitivity in all five tested non-spontaneously growing leukemias. Enhancement of Ara-C toxicity did not occur in the two leukemic cases in which proliferation was not altered by growth factors. Different leukemias with the same proportion of cycling CFU-L (for example patient 1 and 5) were not always equally Ara-C sensitive. This indicated that other than S-phase dependent mechanisms may contribute to the cytotoxicity of Ara-C for instance differences in the level of metabolic resistance. Although not all clonogenic cells were actively cycling, increasing dosages of Ara-C eventually led to a 100% kill. This suggests a cell cycle independent mechanism of toxicity at higher concentrations of Ara-C. Earlier studies indeed reported the additional toxic effects of high-dose Ara-C.[39]

To investigate the S-phase dependent kill of Ara-C in more detail, the distribution of IdUrd$^{+}$ colonies was determined at different inhibiting concentrations of Ara-C (Fig.2, Table 2). Ara-C appeared to be preferentially toxic to IdUrd$^{+}$ progenitors. This supports the hypothesis that actively proliferating CFU-L are more sensitive to Ara-C than slowly- or non-proliferating CFU-L. Since both IdUrd and Ara-C are incorporated into DNA during S-phase, an interaction between the two substances had to be ruled out. In

theory, the chain terminating activity of Ara-C could result in a lower and thereby undetectable number of incorporated IdUrd molecules. At the same time this would lead to substantial DNA damage and presumably cell death. We could demonstrate that Ara-C toxicity was not altered in the presence of 1 μM IdUrd (Fig. 1).

In contrast to the leukemic samples, normal CFU-GM were already actively cycling in the absence of growth factors (median 73.1% $IdUrd^+$) in the experimental design used for this study. This may reflect the real percentage of spontaneous proliferation *in vivo* or more likely may have been caused by for instance endogenous CSF produced by contaminating T-lymphocytes or monocytes. The Ara-C sensitivity of CFU-GM in the presence of IL-3, GM-CSF, and G-CSF was not significantly different from the stimulated CFU-L in this 3-day exposure model. This implies a potential risk for the clinical application of growth factors in conjunction with Ara-C. However, the sensitivity of CFU-GM does not necessarily reflect the sensitivity of the hematopoietic stem cells *in vivo* It is postulated that the majority of normal hematopoietic stem cells are out of cycle *in vivo*. *In vitro* and *in vivo* data thus far suggest that in the presence of growth factors the toxicity of Ara-C towards normal progenitors is not higher than the toxicity of Ara-C alone.[23,40,42,43,44] We found that 10-day Ara-C exposure in the presence of the combination of IL-3, GM-CSF, and G-CSF resulted in a preferential kill of the leukemic versus the normal clonogenic cells *in vitro*.[18]

*In vitro* models provide a tool to investigate regulatory mechanisms in leukemic and normal hematopoiesis. The accuracy of these models can only be proven by *in vivo* studies. Recently, we adapted a protocol for IdUrd administration to patients in order to reach a sufficiently high, but not toxic plasma level to label CFU-L *in vivo*. The preliminary data indicate that also *in vivo* only a minority of CFU-L is actively cycling.[41]

Several recent studies analyzing the influence of G(M)-CSF during remission-induction therapy of AML patients reported conflicting data. Some authors observed a higher percentage of patients achieving complete remission after induction chemotherapy in combination with growth factors.[43] Others found no enhancement of remission rates when growth factors were given concomitantly with chemotherapy.[45] These contrasting data may be explained by this report. Leukemic blasts not responding to the administered CSF are not expected to benefit from the proliferation inducing properties of the growth factor Additional studies need to be performed to establish the beneficial effects of recruitment by growth factors in the treatment of AML patients.

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# Chapter 6

## *Interphase cytogenetics on agar cultures: a novel approach to determine chromosomal aberrations in hematopoietic progenitor cells*

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## Summary

We describe a novel approach to determine the presence of chromosomal aberrations in progenitor cells by in situ hybridization (ISH) on agar cultures. Bone marrow cells of three patients suffering from acute myeloid leukemia (AML) were selected to develop the method. In all three cases, numerical aberrations for chromosome 1 and/or 8 were detected by karyotying and ISH using chromosome-specific centromeric-associated DNA probes. These aberrations were used as markers in this study. After *in vitro* culture of the bone marrow samples in agar, the cells were pretreated to perform ISH. This approach retains the cytological architecture of the agar assay, allowing discrimination between chromosomal aberrations detected in the clonogenic and non-clonogenic cells in culture. With this new technique, the presence of the cytogenetic aberration in clonogenic cells can be shown at the interphase level.

## Introduction

Hematopoietic neoplasms are assumed to be clonal diseases originating from a single cell [1,2] Therefore, monoclonality can be used to further characterize these neoplasms. In the majority of myeloproliferative disorders, progenitors can be cultured *in vitro*. Information about the lineage involvement of these neoplasms may increase understanding of their pathogenesis and response to treatment. Because the origin of clonogenic cells may be difficult to determine by morphology alone,[3] additional techniques have been developed to demonstrate the monoclonal origin of progenitors. The X-chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD), or restriction fragment length polymorphisms (RFLP) of other X-chromosome-linked enzymes, can be used as a genetic marker in heterozygous women.[1] Karyotyping and surface marker expression analysis can also be used [3,4]

Except for the latter technique, all methods analyze colonies that are individually harvested. Apart from being time-consuming, harvesting may lead to sampling error because non-clonogenic cells may be removed in place

of clonogenic cells due to colony overlap. Since karyotyping analyzes dividing cells, cell populations with a selective growth advantage will be favored.[4] Furthermore, within a colony, sufficient proliferative cells must be available. Only relatively large colonies (>100 cells) could be analyzed, although with technical improvements smaller colonies (±50 cells) are being investigated now.[4]

Nonradioactive ISH of chromosome-specific, centromeric-associated DNA probes to interphase cells has become available, determining the copy number of a target chromosome by counting the number of hybridization signals.[5-8] Although this interphase analysis provides only limited information about the karyotype, it enables rapid screening of large numbers of cells.

Until now, few ISH studies have been published in which bone marrow and peripheral blood interphase nuclei from patients suffering from leukemia were analyzed at the total cell level [9-13] Although the detection of structural aberrations is limited using centromeric DNA probes, double-target fluorescent ISH experiments on interphase nuclei have shown that the detection of structural chormosome aberrations is feasible.[14] A two-color ISH approach can be used to demonstrate the *bcr-abl* fusion, which is responsible for the appearance of the Philadelphia chromosome, in interphase cells of chronic myeloid leukemia (CML) patients.[15,16] Also, marker chromosomes can be characterized by using these procedures.

In the present study, the application of interphase cytogenetics at the progenitor cell level is described. For this approach, we developed a method to perform ISH on cells cultured in agar. Since the cytological architecture of the agar assay remains intact, discrimination between chromosomal aberrations detected in both clonogenic and non-clonogenic cells can be detected.

## Materials and methods

Sample selection and preparation Three karyoptyped AML bone marrow samples with numerical chromosome aberrations were selected: monosomy for chromosome 1, trisomy for chromosome 8 and a polyploid karyotype. As a control, the samples were hybridized with a chromosome-specific probe for which no chromosomal aberration was detected. In addition, normal bone marrow cells of a healthy donor served as a control.

Preparations for ISH were made with freshly obtained bone marrow cells. The samples were layered onto Ficoll-Hypaque (specific density 1085 g/ml) (Pharmacia/LKB, Uppsala, Sweden) and centrifuged at 1200 rpm for 20 minutes

Part of the collected low-density cells were washed in phosphate-buffered saline (PBS), fixed in 70% ethanol (-20°C) and stored at -30°C. Shortly before ISH, 5 µl of a cell suspension was dropped onto slides coated with poly-L-lysine (molecular weight 150 to 300 kd) (Sigma, St. Louis, MO), air-dried and heated at 80°C for 1 hour.

The remaining low-density cells were cultured in Iscove's medium (Flow Laboratories, Irvine, Scotland), supplemented with 20% fetal calf serum (FCS), 50 IU/ml penicillin, 50 µg/ml streptomycin (both Flow

Laboratories) and 0 3% (w/v) bacto-agar (Difco, Detroit, MI). The cells were stimulated with the combination of interleukin-3 (IL-3) (40 ng/ml; Sandoz BV, Uden, The Netherlands), granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/m; Sandoz BV) and granulocyte colony-stimulating factor (G-CSF) (5 ng/ml; Behring, Marburg, Germany). Duplicates were cultured in 35- x 10-mm culture dishes (Costar, Cambridge, MA) at 37°C in a fully humidified atmosphere containing 5% $CO_2$.[17] The cells ($2x10^5$) were plated in a minimum volume of 0.85 ml Iscove's medium per dish to make them more accessible to the probes. After 7 days, the total number of clusters (5 to 40 cells) and the total colony number (>40 cells) was counted. The agar cultures were lifted out of the culture dishes and, after division into quarters, spun down on poly-L-lysine-coated slides by cytocentrifugation. Subsequently, the samples were fixed in 70% ethanol, air-dried and stored at 4°C until ISH was performed.

**Tumor cell processing for in situ hybridization** A proteolytic digestion step with pepsin at a concentration of 100 µg/ml in 0.01 M HCl for 20 minutes at 37°C was performed to obtain an optimal recovery of cells and proper removal of cellular protein for improvement of DNA probe and antibody penetration [6,9] Thereafter, the nuclei were postfixed in 4% formaldehyde in PBS for 20 minutes at 4°C. Subsequently, the slides were washed in PBS and $H_2O$ and equilibrated in 2x SSC (0.3 M NaCl, 30 mM Na-Citrate), pH 7.0, for 5 minutes at room temperature.

**DNA probes** The plasmid probes pUC1.77 and D8Z2 (Oncor, Gaithersburg, MD) were used for the detection of target sequences on chromosomes 1 and 8, respectively.[18,19] The probes were labeled by nick-translation with biotin-11-dUTP (Sigma), according to the supplier's instructions

**In situ hybridization** The DNA probes were hybridized to the (tumor-)cell preparations as described before in 60% formamide, 2x SSC and 10% dextran sulfate at a probe concentration of 1 ng/µl hybridization mixture.[9] Ten microliters of the hybridization mixture was applied to the slides under a coverslip (18x18 mm). Denaturation of the probe and targed DNA was performed simultaneously by heating the slides to 70°C in a moist chamber for 2.5 minutes. Hybridization was then performed for 2 to 16 hours at 37°C. The coverslips were removed by immersing the slides in 60% formamide, 2x SSC, pH 7.0. Thereafter, the slides were washed 3 times for 5 minutes in the same buffer at 42°C and subsequently 3 times for 5 minutes in 2x SSC, pH 7.0, at 42°C.

**Immunocytochemistry** Detection of hybridized cells was accomplished using mouse antibiotin (Dakopatts, Glostrup, Denmark) in PBS and 0.05% Tween 20 with 0 5% blocking milk (Boehringer, Mannheim, Germany), followed by an incubation with biotin-labeled horse antimouse (Vector, Burlingame, CA) Next, a final incubation with the ABC complex (avidin-biotin-labeled peroxidase complex) (Vectastain Elite ABC Kit) was performed according to the supplier's instructions. All immunocytochemical steps were performed

for 30 minutes at 37°C. Finally, the DNA probe was visualized with 0.5 mg/ml 3,3-diaminobenzidin tetrahydrochloride (DAB) (Sigma), 0.65% imidazole (Merck, Darmstadt, Germany) and 0.015% $H_2O_2$ (Merck), pH 7.8, in PBS for 5 minutes. Slides were rinsed in distilled water and the signal was amplified with $CuSO_4$ (0.5% in 0.9% NaCl) for 5 minutes at room temperature, washed with distilled water, counterstained with hematoxylin and mounted in Permount (Fisher Scientific, Fair Lawn, NJ).

**Evaluation of ISH results** Using the described ISH procedure, at least 90% of the interphase nuclei showed one, two or three distinct ISH signals in all preparations. Evaluation of the preparations and counting of ISH signals were done by two trained observers according to criteria as described before.[6,9] Minor hybridization signals, which can be recognized by a lower intensity and spot area, and overlapping nuclei were not counted.[20] In the control cells, one ISH signal for a targeted chromosome was found in 5 to 10% of the cells, depending on the efficiency of the ISH procedure, or the co-localization of two ISH signals. The detection of three ISH signals occurred in less than 1% of the normal cells.

Of the cells from suspension preparations, 200 nuclei per slide were counted. The agar preparations were evaluated by counting 200 single cells in agar (representing non-clonogenic cells) and approximately 50 aggregates (both colonies and clusters). Since the aggregates represent clonogenic cells, all the cells in an aggregate should be the same, and the distribution of ISH signals would be an indication for the clonality of the aggregates. Therefore, a cell count of the distribution of ISH signals in an aggregate was not necessary. Moreover, the same criteria for evaluation as described previously could be applied.[9,20] In an aggregate, about 90% showed ISH signals. This means that when more than 90% of the cells within an aggregate contained one ISH signal, it was classified as monosomy. Consequently, of it contained two or three ISH signals, it was classified as disomy and trisomy, respectively

## Results

### Development of the technique

The success rate of the ISH procedure using chromosome-specific DNA probes in interphase nuclei is mainly dictated by the accessibility of the target DNA. Therefore, the effect of different conditions, such as the fixative, pretreatment with agarase and proteolytic digestion, were studied.

To avoid detachment of the agar preparations from the glass slides during pretreatment and ISH denaturation, the slides were coated with poly-L-lysine. Fixation with 70% ethanol was sufficient to avoid loss of material from the slide and to preserve morphology during the permeabilization steps.

To improve the accessibility of the target DNAs and to achieve good penetration of DNA probes and immunological reagents through the agar, a proteolytic digestion with pepsin or agarase was performed. Pretreatment

with pepsin, which removes a large part of the cytoplasmic and nuclear proteins, was shown to be more effective than agarase in improving sensitivity and reproducibility. The most crucial parameter for the success rate of ISH, however, was a reduction of the agar layer thickness. We observed that a reduced volume of agar medium at a technical minimum of 0.85 ml instead of the standard 2.0 ml per dish resulted in about 90% analyzable nuclei.

For the immunological detection assay, fluorochromes or peroxidase-conjugated markers could be applied to detect the biotin-labeled probes The evaluation of ISH results by bright-field microscopy was preferred because of the permanency of the staining. Furthermore, fluorescent techniques are hampered by autofluorescence from the remaining agar.

## Experimental results

The ISH results of the bone marrow samples with the probes for chromosomes 1 and 8 are summarized in Table 1

Normal bone marrow cells from case 1 showed two ISH signals for both centromeric-associated probes (93% for chromosome 1 and 95% for chromosome 8) in the cells from the suspension preparation We also detected a low frequency of cells with one signal for these two probes (5% and 4%). All the aggregates screened ($n = 50$) in the semisolid assay contained a majority of cells with two ISH signals (Fig. 1B). Within aggregates, about 10% of the nuclei showed one signal as a result of overlap of ISH signals. No differences in this respect were seen between chromosome 1 and chromosome 8.

ISH on the interphase nuclei of the cells from the case 2 suspension preparation with a monosomy for chromosome 1, according to its karyotype, showed one ISH signal per nucleus in 71% and two ISH signals in 28%. Analysis of the semisolid assay showed that the majority of cells (about 80%) in the aggregates ($n = 35$) contained one ISH signal per nucleus for chromosome 1 (Fig. 1A). A low percentage of cells (<10%) was found with two ISH signals. The single cells in agar, however, showed 12% of the nuclei with two ISH signals and 88% with one ISH signal. Karyotyping revealed no chromosomal aberration for chromosome 8. Hybridization of the cells from the suspension preparation and the semisolid assay with a chromsome 8-specific probe showed frequency distributions similar to the hybridization results for normal bone marrow cells.

The nuclei of case 3 with a trisomy for chromosome 8 (46,XY/44, XY,-5,-6,+8,+mar1) showed three ISH signals in 94% of the nuclei in the cell suspension preparation. The majority of the nuclei in the aggregates ($n = 64$) contained three ISH signals (Fig. 1C), while the nuclei of the single cells in the agar demonstrated three ISH signals in 73% and two ISH signals in 12%. Furthermore, in this case 11% of the single cells in the agar showed no signal at all. Even after repeated hybridizations, a low frequency of nonreacting cells was observed The results of hybridization with the chromosome-1-specific probe were comparable to those of normal bone marrow cell.

**Table 1** Results from karyotyping and in situ hybridization procedures in single-cell suspension preparations and in the semisolid assay of bone marrow cells of a healthy donor and AML patients

| | | | | Cell suspension | | | | | Semisolid assay | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Single cells in agar | | | | |
| | | | | ISH signals/nucleus (%) | | | | | | ISH signals/nucleus (%) | | | | |
| Case no. | Diagnosis | Karyotype | Probe for chromosome | 0 | 1 | 2 | 3 | 4 | Aggregates[a] | 0 | 1 | 2 | 3 | 4 |
| 1 | normal bone marrow | 46,XY | #1 | 1 | 5 | 93 | 1 | 0 | disomy (n=50)[b] | 2 | 4 | 94 | 0 | 0 |
| | | | #8 | 0 | 4 | 95 | 1 | 0 | disomy (n=50) | 3 | 3 | 92 | 2 | 0 |
| 2 | AML | 46,XY/44,XY,-1,-5,-7, -17,+mar1,+mar2, +2xmar3 | #1 | 0 | 71 | 28 | 1 | 0 | monosomy (n=35) | 0 | 88 | 12 | 0 | 0 |
| | | | #8 | 0 | 9 | 91 | 0 | 0 | disomy (n=23) | 0 | 8 | 90 | 2 | 0 |
| 3 | AML | 46,XY/44,XY,-5,-6 +8,+mar1 | #1 | 0 | 6 | 94 | 0 | 0 | disomy (n=50) | 0 | 6 | 93 | 1 | 0 |
| | | | #8 | 0 | 1 | 5 | 94 | 0 | trisomy (n=64) | 11 | 4 | 12 | 73 | 0 |
| 4 | AML | polyploid 92-96 chromosomes XX | #1 | 0 | 5 | 33 | 18 | 44 | disomy (n=38) | 0 | 2 | 32 | 16 | 50 |

***a*** For evaluation see Materials and methods.

***b*** Number of aggregates (clusters 5-40 cells; colonies >40 cells) analyzed.

ISH on the interphase cells of case 4 with a polyploid karyotype (female, 92-96 chromosomes) showed for chromosome 1 two (33%), three (18%) and four (44%) ISH signals in the single-cell suspension preparation. The single cells in agar also demonstrated the same distribution of ISH signal counts (Table 1), showing three and four ISH signals (Figs. 1F, G). The main population of cells within aggregates showed two ISH signals (Fig. 1E). Within aggregates, however, a low percentage of cells with three or four ISH signals was observed (<20%).

## Discussion

The approach described in this study demonstrates the application of the ISH technique for determining chromosomal aberrations in hematopoietic progenitor cells cultured in a semisolid assay.

The distribution of the ISH signals in the interphase cells of the cell suspension preparations was shown to be similar to those of the single cells in agar in all four cases. This means that these cells are representative of the total

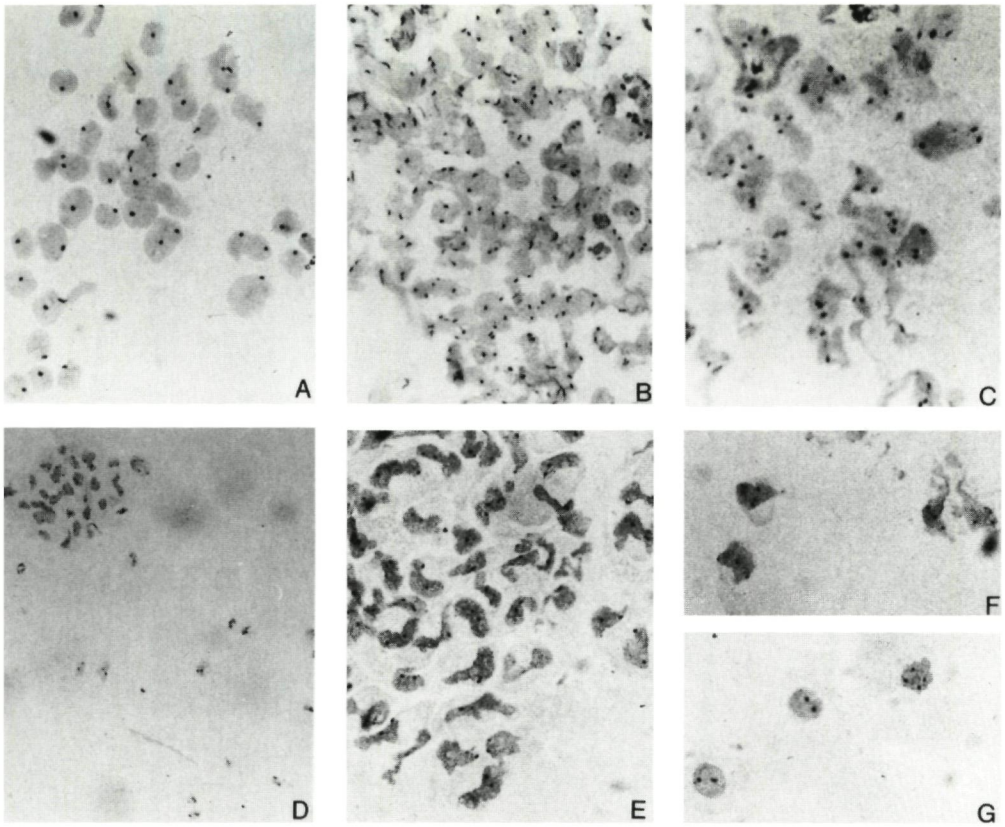


**Figure 1** Application of the in situ hybridization procedure to in vitro cultured progenitor cells from patients with AML.

- **a** Almost all clonogenic cells of case 2 demonstrate one ISH signal per cell for chromosome 1, as detected by ISH on single cells and by karyotyping.
- **b** Part of an aggregate from normal bone marrow (case 1) showing two ISH signals per nucleus.
- **c** Three ISH signals per cell for the chromosome 8 centromeric-associated probe were detected in case 3, in which a trisomy 8 was karyotyped.
- **d** The cytological architecture of the agar assay remains intact.
- **e**, **f** and **g**. Case 4 was karyotyped as polyploid. The aggregates (e) demonstrated two ISH signals per nucleus, whereas single cells in the agar could be recognized showing three (g) or four (f) ISH signals for chromosome 1.

cell population. In cases 2 and 4, a mixed cell population, according to the number of ISH signals for the probe for chromosome 1, was observed. The cells in the aggregates in each of these cases, however, revealed one type of ISH signal. In case 2, a monosomy and in case 4, a disomy could be detected, suggesting monoclonality of the progenitors.

All the analyzed aggregates of cases 2 and 3 contained the clonal marker (monosomy 1 and trisomy 8, respectively), and no disomic aggregates were assessed. Therefore, they all confirmed the leukemic origin. In case 4, which had a polyploid karyotype, the aggregates were diploid. The aggregates demonstrated a mixed population, however-predominantly diploid cells

with a few polyploid cells (<20%). A possible explanation is that the large colonies overlapped the aneuploid cells in this case. This may be the result of the high plating concentration ($8x10^5$ cells per dish) that was required to obtain the colonies.

The major advantage of the ISH approach over karyotyping is that not only metaphase spreads, but also interphase cells can be examined for chromosomal aberrations. Therefore, at least 90% of the cells can be analyzed. Another advantage of the ISH approach is that the cytological architecture of the agar assay remains intact (Fig. 1D). This makes it possible to analyze small clusters containing about 5 to 20 cells; by previous techniques,[4,19] only large colonies coud be analyzed. Furthermore, the aggregates and single cells in agar can be analyzed individually. This makes it possible to discriminate between the clonogenic and non-clonogenic cells in a mixed population. The main advantage of ISH over the G6PD technique is that heterozygous females are not the only patients who can be monitored for the detection of progenitor cells.

ISH, using a specific chromosomal aberration as a marker, can now be applied in addition to cytogenetic analyses to determine the leukemic origin of *in vitro* cultured cells. Furthermore, the ISH technique enables the determination of the origin of blood and bone marrow progenitor cells in patients with myeloproliferative disorders at different stages of the patients' disease. Bone marrow can be maintained in culture for at least 10 days to serve as an autograft in patients with intensive chemo-radiotherapy It is assumed that normal stem cells survive this period better than leukemic stem cells.[21] The ISH technique facilitates the detection of numerical chromosome aberrations in progenitor cells to monitor the *in vitro* culture system.

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# Chapter 7

## *Clonal analysis of progenitor cells by interphase cytogenetics in patients with acute myeloid leukemia and myelodysplasia*

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## Summary

Interphase cytogenetics was used to investigate the clonal origin of bone marrow (BM) cells, peripheral blood (PB) cells, and *in vitro* cultured progenitor cells of five patients with acute myeloid leukemia (AML) and myelodysplasia (MDS). A new in situ hybridization (ISH) technique was used to examine the origin of the progenitor cells.

Two patients with, respectively, trisomy 8 and polyploidy as ISH marker were studied both at presentation and during remission At presentation, the *in vitro* cultured clusters of both cases appeared diploid. Therefore, despite the abnormal growth patterns, the cultured progenitors could have been residual normal cells. Alternatively, they could have originated from a preleukemic clone with a normal karyotype. In both cases abnormal BM and/or PB cells (less than 6%) were detected with ISH during remission, indicating partially or completely clonal remissions in these patients. Both patients have relapsed.

One patient with trisomy 10 as ISH marker was analyzed during myelodysplastic phase and after progression to AML. On both occasions, abnormally appearing clusters were cultured. However, only part of the clusters carried trisomy 10. The presence of a subclone characterized by trisomy 10 and an abnormally growing (pre)leukemic clone without trisomy 10 may explain this observation.

Monosomy 1 and 17 were respectively used as ISH markers in two other AML patients. All *in vitro* cultured clusters carried the numerical abnormality. Long-term liquid cultures of these leukemias were performed for 10-20 days. In both cases, no residual normal clonogenic cells could be detected. Therefore, the selective growth advantage of normal progenitor cells in long-term marrow cultures could not be demonstrated in these two patients with leukemia.

This paper illustrates the usefulness of ISH to study the biology of AML at the clonogenic level during preleukemic phase, active disease, remission,

and under *in vitro* culture conditions. It is a sensitive technique which allows individual analysis of large numbers of small aggregates and single cells in culture.

## Introduction

Acute myeloid leukemia (AML) may be considered as a clonal expansion of one single transformed cell.[1,2] The origin of the leukemic clone may be either a multipotential hematopoietic stem cell (HSC) or a cell restricted to the granulocyte-monocyte pathway.[1,2] In myelodysplastic syndrome (MDS), the abnormal clone is assumed to arise at stem cell level.[3] In the majority of AML/MDS cases, progenitor cells can be cultured *in vitro*.[4-6] The origin of these clonogenic cells may be difficult to assess, since colonies derived from (pre)leukemic progenitors are not always morphologically distinguishable from those derived from normal progenitors.[7]

Several techniques have been developed to determine the clonal origin of hematopoietic cells. In heterozygous females, the X-chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) or restriction fragment length polymorphisms (RFLP) of other X-chromosomal enzymes can be used as a genetic marker.[1,2,8] Karyotyping and surface marker expression analysis offer alternative approaches.[2,9,10] Progenitors of AML patients at presentation are usually derived from the malignant clone. Occasionally, the myeloid colonies appeared to originate from normal stem cells.[7] During remission, in most patients restoration of nonclonal hematopoiesis and repopulation of the marrow by normal stem cells has been observed. Clonal remissions have also been observed.[3,10,11]

The results obtained with long-term bone marrow culture (LTBMC) of BM cells from AML patients additionally support the presence of residual normal stem cells in leukemic patients during active disease. In most cases, leukemic colonies and clusters were no longer detectable after 1 to 4 weeks of culture.[12-14] The few reports about progenitors in MDS suggest the existence of a transformed stem cell either with or without an abnormal karyotype.[5,15]

Recently, we described a method to determine chromosomal aberrations by ISH in myeloid progenitor cells cultured in agar.[16,17] The present study investigates the genetic clonality of myeloid progenitor cells and nucleated cells in five patients with AML and MDS at different stages of their disease.

## Patients and Methods

**Patient selection** Patients with *de novo* AML or AML after a myelodysplastic phase characterized by a numerical chromosomal aberration were selected for this study. AML and MDS were diagnosed according to the French-American-British classification.[18,19] Patient 1 was assessed at diagnosis and in complete remission. He relapsed 6 months after the last analysis (May 1992). Patient 2 presented with a leukemoid reaction during an exacerbation of her ulcerative

colitis. This was followed by a 'spontaneous' remission of 5 months without any antileukemic therapy. Patient 3 was investigated during the myelodysplastic phase and after leukemic transformation. Patients 4 and 5 were studied at presentation of AML. The relevant clinical and hematological data are shown in Table 1.

**Table 1** Clinical data

| Case no | Date | Disease status | Age (yr) | Sex | WBC ($10^9$/l) | Hb (mmol/l) | Platelets ($10^9$/l) | Blood % blasts | BM % blasts | FAB type |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2/88 | AML | 52 | M | 10 5 | 6 4 | 26 | 3 | 85 | M4 |
| | 5/91 | CR | | | 8 6 | 7 5 | 173 | 0 | 0 | |
| | 11/91 | CR | | | 5 4 | 6 9 | 155 | 1 | NA | |
| 2 | 4/87 | 'leukemoid reaction' | 3 | F | 43 6 | 7 7 | 236 | 2 | 36 | |
| | 9/87 | AML | | | 46 9 | 7 4 | 23 | 42 | 80 | M4 |
| | 2/88 | CR | | | 6 7 | 7 8 | 140 | 0 | 2 | |
| 3 | 12/91 | MDS | 54 | M | 7 3 | 7 1 | 30 | 2 | 6 | RAEB |
| | 5/92 | AML | | | 24 7 | 5 7 | 12 | | 33 | M2 |
| 4 | 5/89 | AML | 60 | M | 21 6 | 5 7 | 16 | 27 | 28 | M6 |
| 5 | 2/89 | AML | 58 | M | 8 6 | 5 6 | 29 | 73 | 75 | M2 |

M, *male*, F, *female*, WBC, *white blood cells*; Hb, *hemoglobin*; BM, *bone marrow*; FAB, *French-American-British classification*[18,19], NA, *not available*

**Cell collection, cryopreservation, and thawing** Bone marrow cells were collected, cryopreserved in liquid nitrogen and thawed as described in detail elsewhere.[20] For ISH, cells were fixed in 70% ethanol (-20°C) and stored at -30°C until further use.

**Human recombinant growth factors** Human recombinant interleukin 3 (IL-3) and human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) were kindly donated by Sandoz BV (Uden, The Netherlands). Human recombinant granulocyte colony-stimulating factor (G-CSF) was a kind gift from Behring (Marburg, Germany). Final concentrations of 40 ng/ml, 20 ng/ml, and 5 ng/ml respectively were used and resulted in plateau stimulation.

**Clonogenic assay (CFU-C)** Cells were cultured in Iscove's medium, supplemented with 20% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin (Flow Laboratories, Irvine, Scotland), and 0 3% (w/v) bacto-agar (Difco, Detroit, Michigan) The cells were stimulated with the combination of IL-3, GM-CSF, and G-CSF Duplicates were cultured in 35- x 10-mm culture dishes (Costar, Cambridge, Massachusetts) at 37°C in a fully humidified atmosphere containing 5% $CO_2$ In order to render the cells better accessible for the DNA probes, a reduced volume of 0 85 ml per dish was plated [16] After 7 days, the total number of clusters (5-40 cells) and colonies (>40 cells) was counted A pattern of small (maximum size, 20 cells) or large (maximum size, 40 cells) cluster formation was usually observed After counting, the agar cultures were dried by a method based on a technique described by Baines [21] The cultures were shaken into glucose-phosphate buffer and divided into quarters Each quarter was spun down on a 76- x 26-mm poly-L-lysine (Sigma, St Louis, Missouri, MW 150,000-300,000) coated glass slide by cytocentrifugation Samples were processed for ISH or stained with hematoxylin solution (Merck, Darmstadt, Germany) to evaluate morphology

**Liquid culture** Cells were suspended in Iscove's medium supplemented with 20% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin and a combination of IL-3, GM-CSF, and G-CSF The liquid cultures were maintained in an incubator (37°C, 5% $CO_2$ and fully humidified atmosphere) for a period up to 20 days At regular intervals, part of the cells were collected, washed, and used for clonogenic assay and ISH

**Karyotyping** Metaphase spreads were obtained from BM or PB cells after culturing in RPMI 1640 (Flow Laboratories) for 1 or 24 h Colcemid was present during the last hour of the culture Before fixation in methanol glacial acetic acid (3 1), the cells were exposed to a hypotonic solution (0 075 M KCl) for 15 min Slides were prepared according to routine cytogenetic procedures Karyotyping was performed using the GTG technique

**In situ hybridization** ISH was performed on cell suspensions and dried agar cultures as described before [16] For good penetration of the DNA probes and antibodies, a proteolytic digestion step with pepsin (P7000 Sigma) was performed at a concentration of 100 μg/ml in 0 01 M HCl for 20 min at 37°C The nuclei were post-fixed in 4% formaldehyde in PBS for 20 min at 4°C Ten microliters of the hybridization mixture (60% formamide, 0 6 M NaCl 60 mM sodium citrate (2x SSC), 10% dextran sulphate, and a probe concentration of 1 ng/μl) was applied under a coverslip Denaturation of target DNA and probe was carried out simultaneously at 70°C for 2 5 min in a moist chamber and hybridization was performed for 2-16 h at 37°C Subsequently, the hybridized probes were detected by horseradish peroxidase (HRP) conjugated avidin (Dakopatts, Glostrup, Denmark) Finally, the DNA probe was visualized with 0 5 mg/ml 3,3-diaminobenzidin tetrahydrochloride (DAB, Sigma), 0 65% imidazole (Merck), 0 015% H2O2 (Merck), at pH 7 8 in PBS As controls, the samples were hybridized with a chromosome-specific DNA

probe for which no chromosomal aberration was detected. In these controls, the cells from suspension preparations demonstrated one ISH signal for a target chromosome in 5-10% of the cells, and three ISH signals in <2% of the cells. Of these suspension preparations, 200 nuclei per slide were counted. The agar preparations were evaluated by counting 100 single cells and approximately 50 aggregates. In these controls, at least 90% of the cells contained ISH signals. The percentages of cells containing the euploid number of ISH signals for the control DNA probe-targets varied between 85 and 90%.[16,17]

## Results

### Case 1

**Presentation of AML** At presentation, 12 of 13 analysed BM cell metaphases showed trisomy 8 by karyotyping (Table 2). With ISH, trisomy 8 was found in 71% of the BM cells. A leukemic growth pattern was observed with small clusters consisting of 5-25 immature myeloid cells After hybridization with the probe for chromosome 8, disomy was found in all analyzed clusters, whereas 14% of the single cells in the semi-solid culture had trisomy 8.

**Complete remission** In May 1991, karyotyping revealed only normal BM cells, whereas no metaphases could be detected in the PB. However, by ISH, 6% of the BM cells showed trisomy 8. Both BM and PB exhibited a normal in vitro growth pattern. The colonies appeared morphologically normal and were diploid when analyzed with the probe for chromosome 8. By contrast, in the BM culture, respectively 19% and 4% of the single cells in agar had trisomy and tetrasomy for chromosome 8, whereas the single cells of the PB cultures showed trisomy and tetrasomy 8 in respectively 12% and 2%.

In November 1991 only PB was analyzed. No metaphases were detected. ISH on cell suspension was not performed. A normal growth pattern was observed demonstrating disomy 8 in all aggregates, while part of the single cells in culture had trisomy 8 (7%).

### Case 2

At leukemic presentation, all investigated BM metaphases appeared to be polyploid, containing about 96 chromosomes (Table 2) For ISH analysis, we applied the probe for chromosome 1 in this particular case.

**'Leukemoid reaction'** During 'leukemoid reaction', a normal karyotype was found in nine bone marrow metaphases (Table 2). In contrast, ISH revealed that 4% of the BM cells contained three or four ISH signals with the chromosome 1 probe. The number of colonies in the semi-solid culture was depressed.

Morphology and size of the colonies appeared normal. All analyzed colonies were disomic, whereas 8% of the single cells in agar contained three or four ISH signals.

**Presentation** During overt leukemia, when all analyzed BM metaphases were polyploid, tri- or tetrasomy 1 was found by ISH in 62 % of the BM cells. Only very few colonies and clusters could be cultured in agar. They appeared to have normal morphology and size and were disomic when hybridized with the probe for chromosome 1. Sixty-six percent of the single cells in those cultures were tri- or tetrasomic.

**Complete remission** In February 1988, both normal and polyploid cells were detected by cytogenetic analysis ISH revealed tri- or tetrasomy 1 in 5% of the BM cells. Normal colony numbers were observed. Their morphology and size appeared also normal. All analyzed colonies were disomic, whereas 2% of the single cells in the semi-solid culture were tri- or tetrasomic.

### Case 3

**MDS** Karyotyping of the BM revealed that eight of 32 analyzed metaphases had trisomy 10 (Table 2). With ISH, 26% of the BM cells had trisomy 10. In the PB cells, on which no karyotyping was performed, 14% of the cells showed trisomy 10 by ISH. Both BM and PB cells were growing as clusters consisting of 5-35 immature cells. Although morphologically not distinguishable, both clusters with and without trisomy 10 were observed by ISH in these cultures. The single cells in the BM and PB cultures demonstrated a trisomy for chromosome 10 in 35% and 37%, respectively.

**Presentation** At leukemic presentation, no BM metaphases were detected. By ISH, 16% of the BM cells had trisomy 10. The clusters appeared morphologically identical to the clusters cultured during MDS phase. Trisomy 10 was found in seven of 30 clusters, whereas 8% of the single cells in culture showed trisomy 10.

In the following two cases bone marrow was cultured in liquid in the presence of IL-3, GM-CSF, and G-CSF for up to 20 days to investigate whether residual normal progenitors had a growth advantage compared to the leukemic clone.

### Case 4

**Presentation** At leukemic presentation, karyotyping of BM cells revealed multiple abnormalities in almost all metaphases (Table 3). The probe for chromosome 1 was selected as a marker for ISH. Monosomy 1 was found in 71% of the BM cells. The in vitro growth pattern consisted of small clusters

**Table 2** Bone marrow and blood (case nos 1, 2 and 3)

| | | | | Suspension | | Semi-solid | | |
|---|---|---|---|---|---|---|---|---|
| Case no | Date | Disease status | Cell sample | Karyotype | ISH (%) | ISH colonies/clusters | ISH single cells (%) | CFU-C[c] colonies/clusters |
| 1 | 2/88 | AML | BM | 46,XY/47,XY,+8 ($n$=1/12)[a] (92%) | trisomy 8 (71) | disomy 8 ($n$=17)[b] | trisomy 8 (14) | 0/89 |
| | 5/91 | CR | BM | 46,XY ($n$=32) | trisomy 8 (6) | disomy 8 ($n$=21) | trisomy 8 (19) tetrasomy 8 (4) | 99/34 |
| | 5/91 | CR | PB | no metaphases found | NA | disomy 8 ($n$=18) | trisomy 8 (12) tetrasomy 8 (2) | 33/20 |
| | 11/91 | CR | PB | no metaphases found | NA | disomy 8 ($n$=18) | trisomy 8 (7) | 42/20 |
| 2 | 4/87 | 'leukemoid reaction' | BM | 46,XX ($n$=9) | trisomy 1 (1) tetrasomy 1 (3) | disomy 1 ($n$=17) | trisomy 1 (2) tetrasomy 1 (6) | 12/21 |
| | 9/87 | AML | BM | polyploid (about 96 chromosomes, $n$=6) (100%) | trisomy 1 (18) tetrasomy 1 (44) | disomy 1 ($n$=38) | trisomy 1 (16) tetrasomy 1 (50) | 4/5 |
| | 2/88 | CR | BM | 46,XX/polyploid ($n$=14/2) (13%) | trisomy 1 (2) tetrasomy 1 (3) | disomy 1 ($n$=39) | trisomy1 (1) tetrasomy 1 (1) | 140/14 |
| 3 | 12/91 | MDS | BM | 46,XY/47,XY,+10 ($n$=24/8) (25%) | trisomy 10 (26) | disomy 10 ($n$=6) trisomy 10 ($n$=14) | trisomy10 (35) | 1/116 |
| | | | PB | NA | trisomy 10 (14) | disomy 10 ($n$=18) trisomy 10 ($n$=17) | trisomy10 (37) | 1/43 |
| | 5/92 | AML | BM | no metaphases found | trisomy 10 (16) | disomy 10 ($n$=23) trisomy 10 ($n$=7) | trisomy10 (8) | 18/2160 |

ISH, *in situ hybridization*; BM, *bone marrow*, PB, *peripheral blood*, CR, *complete remission*, NA, *not analyzed*

***a*** Number of mitosis.

***b*** Number of analyzed aggregates.

***c*** Number of CFU-C per 2 x $10^5$ NC (BM) or 8 x $10^5$ NC (PB)

(5-20 cells) consisting of immature cells. All analyzed clusters and 88% of the single cells showed monosomy 1.

**Liquid culture** Liquid culture for 10 days did not significantly influence the number of abnormal cells and clusters. Ninety-one percent of the cells in suspension showed monosomy 1. Again only immature appearing small clusters could be cultured, which all showed monosomy for chromosome 1. The single cells in the semi-solid assay demonstrated in 91% a monosomy 1.

**Table 3** Bone marrow (cases 4 and 5)

| Case no | Days of liquid culture | Suspension: Karyotype | Suspension: ISH (%) | Semi-solid: ISH colonies/clusters | Semi-solid: ISH single cells (%) | CFU-C[c] colonies/clusters |
|---|---|---|---|---|---|---|
| 4 | presentation | 46,XY/44,XY,-1,-5,-7, -17,-20,-21,+mar1, +mar2,+2xmar3 ($n$=2/34)[a] (94%) | monosomy 1 (71) | monosomy 1 ($n$=35)[b] | monosomy 1 (88) | 0/380 |
| | day 10 | NA | monosomy 1 (91) | monosomy 1 ($n$=43) | monosomy 1 (91) | 0/180 |
| 5 | presentation | 46,XY/43,X,3q,+8,+10, -5,-6,-12,-13,-17,-22,-Y +mar1-3 ($n$=1/9) (90%) | monosomy 17 (96) | monosomy 17 ($n$=42) | monosomy 17 (82) | 0/5400 |
| | day 5 | NA | monosomy 17 (99) | monosomy 17 ($n$=38) | NA | 0/5100 |
| | day 10 | NA | monosomy 17 (96) | monosomy 17 ($n$=55) | NA | 0/11000 |
| | day 14 | NA | monosomy 17 (95) | monosomy 17 ($n$=27) | NA | 0/4500 |
| | day 20 | NA | monosomy 17 (96) | monosomy 17 ($n$=26) | monosomy 17 (84) | 0/900 |

ISH, *in situ hybridization*, NA, *not analyzed.*

***a*** Number of mitosis

***b*** Number of analyzed aggregates

***c*** Number of CFU-C per 2 x $10^5$ NC.

## Case 5

**Presentation** Nine out of 10 metaphases showed multiple chromosomal aberrations (Table 3). In this case, monosomy 17 was chosen as ISH marker. Monosomy 17 was detected by ISH in 96% of the BM cells. The clusters consisted of 5-25 immature cells and were all monosomic for chromosome 17. Moreover, monosomy 17 was found in 82% of the single cells in the semi-solid assay.

**Liquid culture** The high plating efficiency allowed in liquid culturing of the leukemic cells up to 20 days. Liquid culture cells and agar cultures (both single cells and clusters) were analyzed at regular intervals. During the whole period the proportion of abnormal cells in liquid remained essentially the same (95-99%). At any time, only aggregates with monosomy 17 were detected by ISH.

## Discussion

Five patients with *de novo* AML or AML after a myelodysplastic phase and abnormal clones characterized by numerical chromosomal abnormalities were subject of this study. In all cases, the chromosomal aberrations observed by cytogenetic analyses could also be detected by ISH. The percentage of abnormal cells was within the same range for both methods taking into account the following considerations. Conventional cytogenetic analysis examines fewer cells than ISH and this may result in a less accurate estimation of the real number of abnormal cells. Furthermore, conventional cytogenetic analysis is performed on dividing cells, which may lead to a selection for the abnormal subpopulation.[9] Non-dividing interphase lymphocytes are included in the ISH analysis and cannot be analyzed by karyotyping. The lymphoid lineage is usually not a part of the abnormal clone and thus may account for the lower incidence of abnormal cells found with ISH.[2]

The detection level of ISH analysis is more sensitive than conventional cytogenetic analysis, especially when trisomic or polyploic markers are used. The number of ISH signals is constant during the whole cell cycle, and in normal diploid cells the number of cells with four ISH signals is maximally 1% (17). The sensivity level of three ISH signals is at the level of 2-4%. ISH analysis detected 4% abnormal cells during the 'leukemoid' reaction of patient 2, while the 9 metaphases revealed 46 normal chromosomes. This result may be regarded as borderline significant, but the significance is further supported by the presence of 8% trisomic or tetrasomic cells in the agar culture of this bone marrow. Remission marrow of patient 1 contained only normal metaphases and 6% abnormal interphase cells as measured with ISH. This patient had persisting abnormal cells in culture 6 months later and consequently relapsed. Similar observations have been made in G6PD and RFLP studies.[7,22]

The ISH marker for abnormal cells was not detected in the cells of the clusters of patients 1 and 2. In both cases the bone marrow and blood cultures contained single cells with numerical abnormalities. The clusters could be the progeny of normal progenitor cells which exhibit an abnormal growth pattern due to influences of adjacent leukemic cells. In the majority of reported cases, myeloid progenitors are part of the leukemic clone at presentation. However, in a few patients, normal CFU-GM have been detected.[7] It is important to realise that the ISH method enables the evaluation of small clusters and single cells in the cultures in situ while the methods based on G6PD isoenzymes or genetic analysis require micromanipulation of the colonies. The latter analyses can only be performed on larger colonies and this may have influenced the results. If the clusters in cases 1 and 2 did originate from normal cells, then the *in vitro* growth pattern and morphology by itself are not sufficient to determine the origin of progenitor cells. An alternative explanation could be that these progenitor cells represent a part of the leukemic clone without the numerical aberration. This concept can be explained by a multistep leukemogenesis with a preleukemic stage; an early step causes clonal proliferation and a later step results in a chromosomal abnormality in descendants of these progenitors.[8,11]

In case 3 the progression from MDS to AML could be observed. The abnormal but identically appearing clusters could be distinguished in two subpopulations. one consisting of progenitors with and the other without trisomy 10 (Table 2). No increase in the percentages of either BM cells or progenitor cells with trisomy 10 was observed when the patient developed AML. Only the plating efficiency of cultured progenitors increased. This observation matches the theory that MDS arises from a transformed progenitor cell and progresses from a preleukemic state to overt leukemia by successive genetic changes.[3,15] In that case, all cultured clusters belonged to a (pre)leukemic clone with abnormal growth characteristics, but only part of this clone obtained the chromosomal aberration trisomy 10.

Patient 4 and 5 were selected because conventional cytogenetic analysis revealed a subpopulation of normal metaphases (Table 3). All cultured clusters from these patients contained the numerical ISH abnormality. This was in agreement with the observed leukemic growth pattern and undifferentiated appearance of the clusters, and confirmed the leukemic origin of these myeloid progenitors. Although in both cases part of the BM cell metaphase spreads and interphase nuclei appeared normal, no residual normal clonogenic cells could be detected after long-term culture in liquid. In the majority of reported cases, long-term marrow cultures provide a selective growth advantage for normal progenitor cells, but in some AML cases, as in patient 4 and 5, persistence of the leukemia has been observed.[12-14]

Forty to 70% of the AMLs and 25 to 50% of the MDSs have numerical chromosomal abnormalities, indicating that a substantial number of patients with AML and MDS can be analyzed by ISH using centromere associated DNA probes.[23,24] Moreover, probes against fusion genes, such as *bcr-abl* gene, have become available.[25] This may further increase the number of patients which can be studied by ISH techniques. By performing ISH on agar cultures, the cytological architecture remains intact. This enables individual analysis of large numbers of aggregates and single cells in the culture. Sample errors are avoided since removal of colonies is not necessary. In contrast to the conventional techniques, not only large colonies but also small clusters can be analyzed. Furthermore, it is less time consuming to investigate large numbers of aggregates. Therefore, ISH may help to clarify the complex biology of MDS and AML both during 'full-blown' situation and clinical remission. In addition, it can be applied to monitor *in vitro* culture systems. Additional studies are needed to explain the nature of small aggregrates of some patients which have a normal diploid ISH signal.

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# Chapter 8

*Summary and conclusions*

## Summary

In both normal and leukemic hematopoiesis, stem cells generate large quantities of more or less differentiated end cells. Leukemic stem cells are held responsible for the maintenance of the leukemic clone. Therefore, the main target of antileukemic therapy is eradication of these leukemic progenitor cells with relative sparing of the normal bone marrow stem cells.

Colony-forming assays are available to culture normal and leukemic clonogenic cells *in vitro*. In this thesis, clonogenic assays were used as models to study the Ara-C sensitivity, growth factor responsiveness, proliferation and clonal origin of leukemic progenitor cells.

*Chapter 1* introduces the normal and leukemic hematopoiesis, the *in vitro* growth of progenitors, the cell cycle, the activity of Ara-C, and interphase cytogenetics. Finally, an outline of the investigation is given.

In *Chapter 2* the cytotoxic effect of the cell cycle-specific agent Ara-C on clonogenic leukemic and normal bone marrow cells was investigated. To overcome kinetic resistance and to increase cytotoxicity, cells were exposed to Ara-C for extended time periods, that is, 5 and 10 days. Prolongation of the Ara-C exposure time from 5 to 10 days increased the toxicity towards the majority of the leukemic clonogenic cells but not towards normal CFU-GM. Significantly more leukemic clonogenic cells than normal CFU-GM were killed after 10-day exposure to Ara-C ($ID_{50}$: $0.8 \pm 0.6 \times 10^{-8}$ M, $n = 9$ and $5.7 \pm 2.8 \times 10^{-8}$ M, $n = 4$ respectively; $p = 0.039$). These results indicate a preferential kill of leukemic versus normal clonogenic cells by prolonged Ara-C exposure.

In *Chapter 3* the stimulation of leukemic clonogenic cells by individual growth factors was related to Ara-C sensitivity. Blasts of AML patients were exposed to IL-3, GM-CSF, G-CSF, and combinations of these hematopoietic factors. Heterogeneity in response was observed resulting in a variable growth factor pattern for individual leukemias. Compared to the toxicity in the absence of growth factors, exposure to Ara-C for 10 days in the presence

of optimal stimulatory factors resulted in a 3- to 1000-fold increase of the Ara-C toxicity in seven patients. The $ID_{50}$ concentrations were 0.48 - 123 x $10^{-8}$ M Ara-C in the absence of growth factors, versus only 0.12 - 0.40 x $10^{-8}$ M Ara-C in the presence of stimulatory factors. In two patients, neither stimulation of the leukemic progenitor cells nor enhancement of the Ara-C toxicity was observed after addition of one or more factors. These results indicate that Ara-C cytotoxicity can be enhanced by individually selected, clonogenic cell growth promoting hematopoietic factors.

*In vitro* suicide techniques are assumed to reflect the proportion of colony-forming cells in S-phase at the time of exposure, but they are not always accurate. In *Chapter* 4, a new IdUrd application was developed to investigate the proliferation of hematopoietic progenitor cells. IdUrd is incorporated into DNA during S-phase and can be visualized by immunoperoxidase containing antibodies. Colony-forming cells in S-phase during the IdUrd exposure were postulated to give rise to $IdUrd^+$ colonies. Toxicity, sensitivity, and IdUrd inactivation studies indicated that progenitor cells could safely be pulse-labeled for 2 hours with 40 μM IdUrd, whereas prolonged labeling with 1 μM was at least feasible for 5 days. The IdUrd pulse-labeling was compared with the golden standard, $^3$H-TdR suicide. Wide confidence intervals of the suicide technique contrasted the small confidence intervals obtained with IdUrd pulse-labeling. In our hands, the IdUrd pulse-labeling was more accurate than the $^3$H-TdR suicide technique.

The prolonged IdUrd labeling technique was used in *Chapter* 5 to investigate whether growth factors influence the kinetics of leukemic clonogenic cells and thus alter the sensitivity to the cell cycle-specific drug Ara-C. Results were compared with the effects on normal CFU-GM. Liquid cultures were performed in the absence and presence of preselected combinations of IL-3, GM-CSF and G-CSF The median percentages of cycling CFU-L, measured by a 3-day IdUrd labeling period, increased significantly ($p = 0.009$) from 8.5% without growth factors to 87.5% with growth factors in five tested non-spontaneously growing leukemias. The Ara-C sensitivity increased median 63-fold (range 14-217). Ara-C appeared to be preferentially cytotoxic to CFU-L going through S-phase. Two spontaneously growing leukemias demonstrated a high proportion of CFU-L in S-phase (93% and 99.3%) and were highly Ara-C sensitive regardless whether exposed to the combination of IL-3, GM-CSF, and G-CSF or not. Different leukemias with a comparable proportion of cycling CFU-L were not equally Ara-C sensitive, suggesting other than cell cycle-specific mechanisms of toxicity. The three tested normal bone marrows showed a more uniform response pattern. Even in the absence of growth factors, 70%-80% of CFU-GM were cycling. This increased to 100% in the presence of the combination of IL-3, GM-CSF, and G-CSF and resulted in median 4.3-fold (range 1.4-5.3) enhancement of Ara-C toxicity. Our data indicate that the increment of cycling leukemic clonogenic cells by growth factors may help to overcome the kinetic resistance to Ara-C in AML patients.

Leukemic blast samples may contain residual normal progenitor cells. Morphology alone can not always discriminate between leukemic and normal aggregates. Therefore, additional techniques like karyotyping, immunophenotyping, and G6PD analysis (X-linked polymorphism) have been developed to establish the leukemic origin of progenitor cells. These techniques have their intrinsic limitations. In *Chapter 6* a novel in situ hybridization (ISH) technique was developed to determine the presence of chromosomal aberrations in progenitors in agar cultures. This technique retains the cytological architecture, allowing evaluation of large and small colonies and non-clonogenic cells in culture Not only metaphase cells, but also interphase cells could be evaluated.

In *Chapter 7* this new ISH technique was applied to investigate the clonal origin of bone marrow (BM) cells, peripheral blood (PB) cells, and in vitro cultured progenitor cells of five patients with AML and MDS with an abnormal chomosomal pattern. Patients were studied during preleukemic phase, active disease, and remission. All (pre)leukemic samples demonstrated abnormal growth at presentation (hypoplastic or small clusters). In two cases the ISH marker was not present in the aggregates, suggesting residual normal progenitors or a leukemic subclone with a karyotype without the ISH marker. In two cases a low percentage (maximally 6%) of abnormal BM and/or PB cells was detected with ISH during remission. Consequently, these remissions could have been partially or completely clonal. In one case, typed as M6, the percentage of BM cells with the ISH marker was much higher (71%) than the amount of blasts (28%) This indicated that at least part of the normoblasts belonged to the abnormal clone, suggesting a multipotent stem cell origin. Long-term liquid cultures of two leukemias were performed for 10-20 days. In both cases, no residual normal clonogenic cells could be detected. Therefore, the reported selective growth advantage of normal progenitor cells in long-term marrow cultures can not be demonstrated in all patients with leukemia.

## Conclusions

- Prolonged exposure to Ara-C may overcome kinetic resistance of slowly proliferating leukemic stem cells.
- Induction of leukemic stem cells into proliferation with individually selected appropriate hematopoietic growth factors may overcome kinetic resistance.
- The new technique utilizing IdUrd to measure proliferation is a more accurate technique to assess proliferation in clonogenic cells.
- The development of a new ISH technique allows detection of numerical chromosomal aberrations in clonogenic cells. This technique facilitates research on leukemic stem cells.

## Samenvatting

Normale en leukemische hematopoietische stamcellen zorgen voor de produktie van min of meer gedifferentieerde eindcellen. De leukemische stamcellen zijn verantwoordelijk voor het voortbestaan van de leukemische kloon. Het belangrijkste doel van antileukemische therapie is het uitroeien van deze leukemische stamcellen en tegelijk het behoud van voldoende normale beenmergstamcellen.

Normale en leukemische progenitorcellen kunnen *in vitro* gekweekt worden middels clonogene assays Deze assays zijn in dit proefschrift gebruikt als modellen om de Ara-C gevoeligheid, de groeifactor gevoeligheid, de proliferatie en de clonale origine van leukemische progenitorcellen te bestuderen.

*Hoofdstuk 1* geeft uitleg over de normale en leukemische hematopoiese, de *in vitro* groei van progenitorcellen, de celcyclus, de werking van Ara-C en interfase cytogenetica. Tot slot wordt een overzicht van het onderzoeksplan gegeven.

Het cytotoxische effect van het S-fase specifieke middel Ara-C op leukemische en normale clonogene cellen wordt bestudeerd in *Hoofdstuk 2*. Om kinetische resistentie te kunnen overwinnen werden de cellen langdurig aan Ara-C blootgesteld, d.w.z. gedurende 5 en 10 dagen. Verlenging van de Ara-C expositieduur van 5 naar 10 dagen verhoogde de toxiciteit t.a.v de meerderheid van de leukemieën, maar niet t.o.v. de normale CFU-GM. Significant meer leukemische clonogene cellen dan normale CFU-GM werden gedood tijdens de 10 dagen blootstelling aan Ara-C ($ID_{50}$: $0.8 \pm 0.6 \times 10^{-8}$ M, $n = 9$ en $5.7 \pm 2.8 \times 10^{-8}$ M, $n = 4$ respectievelijk; $p = 0.039$). Deze resultaten suggereren dat leukemische clonogene cellen gevoeliger zijn voor langdurige blootstelling aan Ara-C dan normale clonogene cellen.

De relatie tussen Ara-C toxiciteit en groeifactor gevoeligheid werd onderzocht in *Hoofdstuk 3*. Blasten van AML patienten werden gestimuleerd met IL-3, GM-CSF, G-CSF en combinaties van deze 3 factoren. Elke leukemie bleek zijn eigen individuele gevoeligheidspatroon te hebben. Tien dagen Ara-C blootstelling in aanwezigheid van optimaal stimulerende factoren deed de Ara-C toxiciteit 3 tot 1000 maal toenemen in 7 patienten in vergelijking met de toxiciteit in afwezigheid van groeifactoren. De $ID_{50}$ Ara-C concentraties waren $0.48 - 123 \times 10^{-8}$ M in de afwezigheid van groeifactoren versus $0.12 - 0.40 \times 10^{-8}$ M in de aanwezigheid van stimulerende groeifactoren. In 2 patienten konden de leukemische progenitorcellen niet gestimuleerd worden door groeifactoren. De Ara-C gevoeligheid nam in deze gevallen niet toe na toevoeging van groeifactoren. Bovenstaande resultaten wijzen erop dat Ara-C toxiciteit verhoogd kan worden door individueel geselecteerde, clonogene celgroei bevorderende groeifactoren.

Van *in vitro* suicide technieken wordt verondersteld dat ze het percentage clonogene cellen in S-fase ten tijde van de expositie weergeven. Deze testen blijken niet altijd even accuraat. Een nieuwe IdUrd toepassing om de proli-

feratie van hematopoietische progenitors vast te stellen wordt beschreven in Hoofdstuk 4. IdUrd wordt ingebouwd in DNA gedurende de S-fase en kan zichtbaar worden gemaakt door aan specifieke antilichamen gekoppeld peroxidase. Uit clonogene cellen, die in S-fase waren gedurende de IdUrd blootstelling, ontstaan IdUrd$^+$ colonies. Bestudering van toxiciteit, sensitiviteit en IdUrd inaktivatie toonde aan dat progenitorcellen konden worden gelabeld gedurende een korte incubatie van 2 uur met 40 µM IdUrd, terwijl langdurige labeling met 1 µM IdUrd gedurende tenminste 5 dagen mogelijk was. De IdUrd puls labeling werd vergeleken met de gouden standaard, de thymidine suicide techniek De betrouwbaarheidsintervallen van de IdUrd labeling waren kleiner dan die van de suicide techniek. De IdUrd puls labeling was in onze handen nauwkeuriger dan de suicide techniek.

In Hoofdstuk 5 werd langdurige IdUrd labeling toegepast om te onderzoeken of groeifactoren de kinetiek van leukemische clonogene cellen veranderen en daarmee ook de gevoeligheid voor het S-fase specifieke cytostaticum Ara-C. De resultaten werden vergeleken met de effekten op normale CFU-GM. Cellen werden gekweekt in de aan- en afwezigheid van geselecteerde combinaties van IL-3, GM-CSF en G-CSF. Bij 5 niet spontaan groeiende leukemieën was na 3 dagen IdUrd labeling het mediane percentage CFU-L in S-fase 8.5% in de afwezigheid van groeifactoren en 87.5% in de aanwezigheid van groeifactoren ($p = 0.009$). De Ara-C gevoeligheid nam mediaan 63 maal toe (spreiding 14-217) wanneer groeifactoren aanwezig waren. Ara-C was bij voorkeur toxisch voor CFU-L in S-fase. Twee spontaan groeiende leukemieën vertoonden een hoog percentage CFU-L in S-fase (93% en 99.3%) en waren zeer gevoelig voor Ara-C zowel in aan- als afwezigheid van de combinatie van IL-3, GM-CSF en G-CSF. Verschillende leukemieën met een vergelijkbaar percentage CFU-L in S-fase waren niet even gevoelig voor Ara-C. Dit suggereert dat ook andere dan celcyclus specifieke mechanismen van Ara-C toxiciteit een rol kunnen spelen. Het beenmerg van 3 normale donoren vertoonde een meer uniforme reaktie. Zonder exogene groeifactoren waren 70% tot 80% van de CFU-GM in S-fase. In de aanwezigheid van de combinatie van IL-3, GM-CSF en G-CSF nam dit toe tot 100% en nam de Ara-C toxiciteit mediaan 4.3 maal toe (spreiding 1.4-5.3). De resultaten geven aan dat de door groeifactoren veroorzaakte toename van leukemische clonogene cellen in S-fase de kinetische resistentie voor Ara-C kan doen verminderen in AML patienten

Leukemisch beenmerg kan naast blasten ook residuale normale progenitorcellen bevatten. Morfologisch kunnen leukemische aggregaten niet altijd goed onderscheiden worden van normale aggregaten. Om die reden zijn aanvullende technieken ontwikkeld zoals karyotypering, immuno-fenotypering en G6PD analyse (X-linked polymorfisme). Bovengenoemde technieken hebben hun beperkingen. In Hoofdstuk 6 wordt een nieuwe in situ hybridisatie (ISH) techniek beschreven waardoor chromosomale afwijkingen in progenitors in agarbodems vastgesteld kunnen worden. De cytologische architectuur blijft onbeschadigd waardoor zowel grote als kleine colo-

nies en niet clonogene losse cellen beoordeeld kunnen worden. Niet alleen metafase cellen, maar alle interfase cellen kunnen worden bestudeerd.

Deze nieuwe ISH techniek werd toegepast in *Hoofdstuk 7* om de clonale origine vast te stellen van beenmergcellen, perifere bloedcellen en *in vitro* gekweekte progenitorcellen van 5 patienten met AML of MDS met een afwijkend chromosomenpatroon. De patienten werden bestudeerd tijdens preleukemische fase, aktieve ziekte en remissie. Alle (pre)leukemieën vertoonden een abnormaal groeipatroon (hypoplastisch of kleine clusters). De aggregaten van 2 patienten bevatten de ISH marker niet. Dit suggereert de aanwezigheid van residuale normale progenitors of een leukemische subkloon met een karyotype zonder de ISH marker. Een klein percentage (maximaal 6%) abnormale beenmerg en/of bloedcellen kon worden aangetoond in 2 leukemieën in remissie. Deze remissies zijn blijkbaar geheel of gedeeltelijk klonaal geweest. Het beenmerg van een type M6 leukemie bevatte meer cellen met de ISH marker (71%) dan het aantal blasten (28%). Dit wijst erop dat ten minste een deel van de normoblasten tot de abnormale kloon behoorden, hetgeen past bij een multipotente stamcel origine. Cellen van 2 patienten met leukemie werden gedurende 10 tot 20 dagen doorgekweekt. In beide kweken konden geen residuale normale clonogene cellen worden aangetoond. Het gerapporteerde groeivoordeel van normale progenitors in lange termijn kweken kan kennelijk niet gedemonstreerd worden in elke leukemie.

## Conclusies

- Langdurige blootstelling aan Ara-C kan de kinetische resistentie van langzaam prolifererende leukemisch stamcellen verminderen.
- Proliferatie-induktie van leukemische stamcellen met individueel aangepaste hematopoietische groeifactoren kan de kinetische resistentie reduceren.
- De nieuwe Idurd techniek maakt een meer accurate vaststelling van de proliferatie van clonogene cellen mogelijk.
- Door de ontwikkeling van een nieuwe ISH toepassing zijn numerieke chromosomale afwijkingen te constateren in clonogene cellen. Dit vereenvoudigt leukemisch stamcel onderzoek.

## Dankwoord

Zeer veel personen hebben hun bijdrage geleverd aan de totstandkoming van dit proefschrift. Zoals velen voor mij reeds hebben vastgesteld is het onmogelijk iedereen bij naam te noemen en dank ik bij voorbaat alle 'onbenoemden'.

Als eerste wil ik mijn promotor Theo de Witte noemen Onze allereerste samenwerking dateert reeds uit 1985. Mijn interesse voor de hematologie is toen ontstaan en resulteerde in een vervolg na mijn afstuderen. Door jouw persoonlijke inzet en het telkens opnieuw vinden van financiële gaatjes is het mogelijk geweest dit onderzoek te doen Alhoewel het tempo de laatste jaren, noodgedwongen, langzaam was, bleef je volhouden mij te stimuleren en is het uiteindelijk toch gelukt.

Met 2 linkerhanden stapte ik het lab in om er naderhand op z'n minst met 1 rechterhand weer uit te komen. De kunst van het steriel kweken en logisch plannen van proeven heb ik van Everdien Koekman geleerd. Het geduldig turen in de microscoop om colonies en clusters te discrimineren heb ik onder de knie kunnen krijgen met hulp van de helaas te vroeg overleden Gemma Blankenborg. Van Petra Muus kreeg ik praktische tips aangezien zij al voor mij uitgebreid leukemieën aan Ara-C had blootgesteld.

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Pino Poddighe, een deel van mijn onderzoek is eigenlijk 'ons' onderzoek. Het leek met horten en stoten te gaan en zat niet altijd mee. Toch zou ik onze 'joint venture' overall als geslaagd willen betitelen. Ook jouw mede-labgenoten pathologische anatomie dank ik voor de samenwerking.

Hans Wessels was altijd bereid manuscripten en proefopzetten kritisch te beoordelen, waarbij soms door het rood de oorspronkelijke tekst niet meer terug te vinden was. De statistische onderbouwing van de behaalde resultaten werd verantwoord gemaakt door Jan Boezeman.

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## Curriculum Vitae

Op 18 maart 1962 werd de schrijfster van dit proefschrift geboren in het Gemeente Ziekenhuis te Arnhem. Het Atheneum B diploma werd in 1980 behaald aan het Liemers College te Zevenaar. Aansluitend studeerde zij geneeskunde aan de Katholieke Universiteit te Nijmegen. In 1985 vervulde zij een keuzevakstage hematologie onder leiding van de toen nog Dr. T.J.M. de Witte. Het artsexamen werd behaald in 1987. Na aanvankelijk als vrijwilligster te hebben gewerkt op de afdeling hematologie kon zij vanaf februari 1988 starten met het onderzoek waarop dit proefschrift is gebaseerd (hoofden successievelijk Prof. Dr. C.A.M. Haanen en Prof. Dr. T.J.M. de Witte). Tijdens de onderzoeksperiode was zij tevens coördinator van diverse klinische trials met groeifactoren bij vnl. AML en MDS patienten.

Per 1 januari 1992 begon zij haar opleiding interne geneeskunde in het Rijnstate Ziekenhuis (locatie Diaconessenhuis) te Arnhem (opleiders achtereenvolgens Dr J M. Werre en Dr. L Verschoor). Per februari 1995 keerde zij terug naar haar 'roots' nadat het nieuwe Rijnstate ZH op de voormalige GZ locatie in gebruik werd genomen. Sinds 1 november 1995 vervolgt zij haar opleiding in het Radboud Ziekenhuis te Nijmegen (opleider Prof. Dr. J.W.M. van der Meer).